



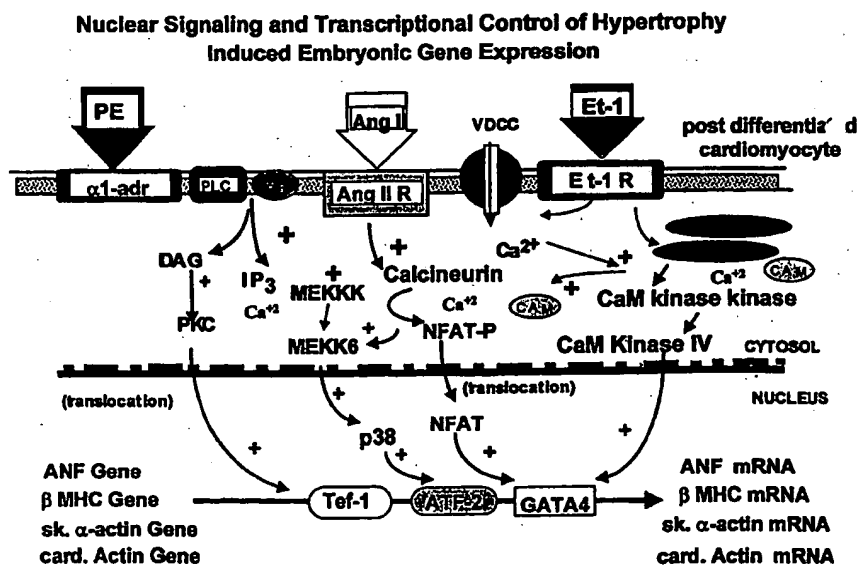
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(54) Title: **TRANSGENIC ANIMAL MODELS FOR CARDIAC HYPERTROPHY AND USES THEREOF**

## (57) Abstract

Transgene constructs for generating transgenic animals, wherein the transgene encodes a gene product which modulates transcription of a hypertrophy-sensitive gene, are provided. Further provided are recombinant vectors comprising the transgenes of the invention. Further provided are transgenic animals generated using the transgene constructs. Further provided are enzyme-based, cell-based, and whole-animal-based assays for detecting substances having therapeutic activity toward cardiac hypertrophy. Further provided are compositions comprising substances which modulate levels of active product of a hypertrophy-sensitive gene. Further provided are methods of treating cardiac hypertrophy.

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**TRANSGENIC ANIMAL MODELS FOR CARDIAC HYPERTROPHY AND USES THEREOF****CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims benefit of U.S. Provisional Application Serial No. 60/062,864, filed October 16, 1997, U.S. Provisional Application Serial No. 60/065,178, filed November 10, 1997, U.S. Provisional Application Serial No. 60/081,853, filed April 15, 1998, and U.S. Patent Application No. 09/061,417, filed April 16, 1998.

**STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER  
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**TECHNICAL FIELD**

The present invention relates to transgenic animal models for cardiac hypertrophy. It further relates to the use of such transgenic animals to detect substances with therapeutic activity toward cardiac hypertrophy. It further relates to assays to detect substances for use in treating cardiac hypertrophy. It further relates to methods for treating cardiac hypertrophy.

**BACKGROUND ART****Cardiac hypertrophy**

Cardiac hypertrophy is an adaptive response of the heart to virtually all forms of cardiac disease, including those arising from hypertension, mechanical load, myocardial infarction, cardiac arrhythmias, endocrine disorders, and genetic mutations in cardiac contractile protein genes. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to dilated cardiomyopathy, heart failure, and sudden death. In the United States, approximately half a million individuals are diagnosed with heart failure each year, with a mortality rate approaching 50%.

Despite the diverse stimuli that lead to cardiac hypertrophy, there is a prototypical final molecular response of cardiomyocytes to hypertrophic signals that involves an increase in cell size and protein synthesis, enhanced sarcomeric organization, up-regulation of fetal cardiac genes, and induction of immediate-early genes, such as *c-fos* and *c-myc*.  
5 See, Chien et al. (1993) *Ann. Rev. Physiol.* 55:77-95; and Sadoshima and Izumo (1997) *Ann. Rev. Physiol.* 59:551-571. The causes and effects of cardiac hypertrophy have been extensively documented, but the underlying molecular mechanisms that couple hypertrophic signals initiated at the cell membrane to the reprogramming of cardiomyocyte gene expression remain poorly understood. Elucidation of these mechanisms is a central  
10 issue in cardiovascular biology and will be critical for designing new strategies for prevention or treatment of cardiac hypertrophy and heart failure.

Numerous studies have implicated intracellular  $\text{Ca}^{2+}$  as a signal for cardiac hypertrophy. In response to myocyte stretch or increased loads on working heart preparations, intracellular  $\text{Ca}^{2+}$  concentrations increase (Marban et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:6005-6009; Bustamante et al. (1991) *J. Cardiovasc. Pharmacol.* 17:S110-S113; and Hongo et al. (1995) *Am J. Physiol.* 269:C690-C697, consistent with a role of  $\text{Ca}^{2+}$  in coordinating physiologic responses with enhanced cardiac output. A variety of humoral factors, including angiotensin II (AngII), phenylephrine (PE), and endothelin-1 (ET-1), which induce the hypertrophic response in cardiomyocytes, also  
15 share the ability to elevate intracellular  $\text{Ca}^{2+}$  concentrations. Karliner et al. (1990) *Experientia* 46:81-84; Sadoshima and Izumo (1993) *Circ. Res.* 73:424-438; Sadoshima et al. (1993) *Cell* 75:977-984; and Leite et al. (1994) *Am. J. Physiol.* 267:H2193-H2203.

Hypertrophic stimuli result in reprogramming of gene expression in the adult myocardium, such that genes encoding fetal protein isoforms like  $\beta$ -myosin heavy chain (MHC) and  $\alpha$ -skeletal actin are up-regulated, whereas the corresponding adult isoforms,  $\alpha$ -MHC and  $\alpha$ -cardiac actin, are down-regulated. The natriuretic peptides, atrial natriuretic factor (ANF), and b-type natriuretic peptide (BNP), which decrease blood pressure by vasodilation and natriuresis, are also rapidly up-regulated in the heart in response to hypertrophic signals. Komuro and Yazaki (1993) *Ann. Rev. Physiol.* 55:55-75.  
25 The mechanisms involved in coordinately regulating these cardiac genes during hypertrophy are unknown, although binding sites for several transcription factors, including serum response factor (SRF), TEF-1, AP-1, and Sp1, are important for activation  
30

of fetal cardiac genes in response to hypertrophy. Sadoshima and Izumo (1993); Sadoshima et al. (1993); Kariya et al. (1994) *J. Biol. Chem.* 269:3775-3782; Karns et al. (1995) *J. Biol. Chem.* 270:410-417; and Kovacic-Milivojevic et al. (1996) *Endocrinol.* 137:1108-1117. Most recently, the cardiac-restricted zinc finger transcription factor  
5 GATA4 has also been shown to be required for transcriptional activation of the genes for Ang II type 1a receptor and  $\beta$ -MHC during hypertrophy. Herzig et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:7543-7548; Hasegawa et al. (1997) *Circulation* 96:3943-3953; and Molken-  
10 tentin and Olson (1997) *Circulation* 96:3833-3835.

A number of intracellular signaling pathways have been implicated in transduction  
10 of hypertrophic stimuli. For example, occupancy of the cell surface receptors for AngII, PE, and ET-1 leads to activation of phospholipase C, resulting in the production of diacylglycerol and inositol triphosphate, which in turn results in mobilization of intracellular  $\text{Ca}^{2+}$  and activation of protein kinase C (PKC). Sadoshima and Izumo (1993); Yamazaki et al. (1996) *J. Biol. Chem.* 271:3221-3228; and Zou et al. (1996) *J. Biol. Chem.*  
15 271:33592-33597. There is also evidence that the Ras and mitogen-activated protein (MAP) kinase pathways are transducers of hypertrophic signals. Thorburn et al. (1993) *J. Biol. Chem.* 268:2244-2249; and Force et al. (1996) *Circ. Res.* 78:947-953. The extent to which these signaling pathways are coordinated during cardiac hypertrophy is unknown. However, all of these pathways are associated with an increase in intracellular  $\text{Ca}^{2+}$ ,  
20 consistent with a central regulatory role of  $\text{Ca}^{2+}$  in coordinating the activities of multiple hypertrophic signaling pathways.

In B and T cells, the  $\text{Ca}^{2+}$ , calmodulin-dependent phosphatase calcineurin has been shown to link intracellular signaling pathways that result in elevation of intracellular  $\text{Ca}^{2+}$  with activation of the immune response. Calcineurin regulates immune response genes  
25 through dephosphorylation of a family of transcription factors known as NF-ATs (nuclear factors of activated T cells). Rao et al. (1997) *Ann. Rev. Immunol.* 15:707-747. Once dephosphorylated by calcineurin, NF-AT transcription factors translocate to the nucleus and directly activate immune response genes. Flanagan et al. (1991) *Nature* 352:803-807; Loh et al. (1996) *Mol. Cell. Biol.* 16:3945-3954; and Loh et al. (1996) *J. Biol. Chem.*  
30 271:10884-10891. The immunosuppressant drugs cyclosporin A (CsA) and FK506 suppress the immune response by inhibiting calcineurin's ability to activate NF-AT

transcription factors. Shaw et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:11205-11209; Loh et al. (1996) *J. Biol. Chem.* 271:10884-10891.

There are no spontaneous mouse mutations with sufficient similarities to cardiac hypertrophy to be useful as experimental models. A transgenic rodent line has been produced that overexpresses calmodulin under the control of the human atrial natriuretic factor gene. Gruver et al. (1993) *Endocrinology* 133:376-388. In these mice, developmental overexpression of CaM in mouse cardiomyocytes produced a markedly exaggerated cardiac growth response, characterized by the presence of cardiomyocyte hypertrophy in regions demonstrated to overexpress CaM and by cardiomyocyte hyperplasia, apparent at early developmental stages. However, the expression of calmodulin in these animals is constitutive and is thus not reflective of physiological conditions in humans. Transgenic mice have been created in which a reporter gene is regulated by a tetracycline-controlled transactivator (tTA), which in turn is under the transcriptional control of 2.9 kb of 5' flanking sequence from the rat  $\alpha$ -myosin heavy chain gene. Yu et al. (1996) *Circ. Res.* 79:691-697.

Current medical management of cardiac hypertrophy includes the use of three types of drugs: calcium channel blocking agents,  $\beta$ -adrenergic blocking agents, and disopyramide. Kikura and Levy (1995) *Int. Anesthesiol. Clin.* 33:21-37. Therapeutic agents for heart failure include angiotensin II converting enzyme (ACE) inhibitors and diuretics. Other pharmaceutical agents which have been disclosed for treatment of cardiac hypertrophy include angiotensin II receptor antagonists (U.S. Patent No. 5,604,251); and neuropeptide Y antagonists (International Patent Publication No. WO 98/33791). Despite currently available pharmaceutical compounds, prevention and treatment of cardiac hypertrophy, and subsequent heart failure, continue to present a therapeutic challenge.

Thus, there is a need for the development of new pharmacologic strategies for prophylaxis and treatment of cardiac hypertrophy in humans. In order to develop such strategies, there is a need for animal models which accurately reflect the pathological profile of the disease, to allow identification of novel targets for therapeutic intervention. In addition, there is a need for novel assays that allow identification of potential new therapeutic agents to treat cardiac hypertrophy.

## SUMMARY OF THE INVENTION

The present invention provides non-human animal models for cardiac hypertrophy. Such models are useful for identifying additional targets for treatment of cardiac hypertrophy, and for testing substances for efficacy in treating cardiac hypertrophy. In one aspect, the invention provides a transgenic non-human animal comprising as a transgene a polynucleotide which, when introduced at an early stage into a fertilized egg or embryo of a mammalian non-human animal, can be functionally integrated into the genome thereof, thereby to produce a transgenic animal, characterized in that the polynucleotide comprises a nucleotide sequence encoding a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a cardiac hypertrophic signal, in combination with control sequences, including, for example, a promoter, which direct and regulate expression of the gene in somatic cells of the transgenic animal, particularly in cardiomyocytes.

The present invention encompasses a transgenic animal comprising as a transgene a polynucleotide encoding a gene product that modulates transcription of at least one gene expressed in cardiomyocytes in response to a cardiac hypertrophic signal, wherein the animal has a substantially increased probability of spontaneously developing symptoms of hypertrophic cardiomyopathy or similar heart dysfunction.

The present invention further encompasses a transgenic animal comprising as a transgene a polynucleotide encoding a gene product that modulates transcription of at least one gene expressed in cardiomyocytes in response to a cardiac hypertrophic signal, wherein the animal has a substantially decreased probability of spontaneously developing symptoms of hypertrophic cardiomyopathy or similar heart dysfunction.

Transgenic animals of the invention are useful in identifying novel targets for prophylaxis and therapy of cardiac hypertrophy and cardiac hypertrophy-induced dysfunctions. These animals are also useful for investigative purposes, for examining signal transduction pathways involved in response to hypertrophic signals. These animals are further useful to screen for potential therapeutic agents for treatment and/or prophylaxis of cardiac hypertrophy.

Preferably, the polynucleotide used to generate a transgenic animal of the invention is a recombinant DNA construct in which the promoter and at least some control

sequences direct expression of an operably linked coding sequence. In some embodiments, the promoter and control sequences are expressed in a cardiomyocyte-specific manner. The coding sequence is fused to a downstream segment comprising at least a fragment of a eukaryotic gene sequence effective to provide signals for terminating transcription and for controlling processing of transcribed RNA during expression of the encoded gene product. In one embodiment, the promoter is derived from an  $\alpha$ -myosin heavy chain gene ( $\alpha$ -MHC). In other embodiments, the transcriptional control sequences comprise elements which confer inducible expression on the operably linked coding sequence. In some embodiments, the transgenic animal comprises a transgene which encodes a polypeptide which modulates transcription of at least one cardiac hypertrophy-sensitive gene. In these embodiments, the polypeptide can comprise a wild-type amino acid sequence or a mutant amino acid sequence such that the polypeptide has altered function, such as an altered enzymatic function or has altered regulatory properties, including, for example, a dominant negative mutant. In other embodiments, the transgene-encoded gene product is an antisense polynucleotide. In yet other embodiments, the transgene-encoded gene product is a ribozyme.

The constructs are introduced into animal embryos using standard techniques such as microinjection or embryonic stem cells. Cell culture-based models can also be prepared by various methods. For example, cells can be isolated from the transgenic animals or prepared from established cell cultures using the same constructs with standard cell transfection techniques.

The invention further provides a method for producing a transgenic animal, for example a mouse or other rodent, having either a substantially increased probability of spontaneously developing hypertrophic cardiomyopathy or similar heart dysfunction or a substantially decreased probability of spontaneously developing hypertrophic cardiomyopathy or similar heart dysfunction, said method comprising incorporating a polynucleotide as specified above into the genome of a non-human animal such that it is functionally integrated therein. At least in preferred embodiments the nucleic acid is a recombinant DNA which is introduced into a fertilized egg or embryo of the animal in order to become functionally integrated into the genome thereof, said recombinant DNA being characterized in that it contains a segment comprising a gene sequence coding a gene product which of modulates transcription of at least one gene that is expressed in



cardiomyocytes in response to a hypertrophic signal, or for a derivative of such a gene product, which segment is fused to an upstream segment comprising promoter and at least some control sequences effective to direct and regulate expression of said coding, and also, preferably, to a downstream segment comprising a eukaryotic gene sequence or fragment effective to provide signals for termination of transcription and for controlling processing of the transcribed RNA.

Constructs for use in generating transgenic animals can be constructed such that expression of the transgene can be constitutive or inducible. A polynucleotide, for use in generating a transgenic animal, comprises a nucleotide sequence which encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. In one aspect, the nucleotide sequence which encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal is constitutively expressed. In another aspect, the gene which encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal is expressed in a regulatable manner. In another aspect, the gene which encodes a gene product that modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal comprises a portion which encodes a dominant negative mutant of a polypeptide.

In one aspect of the invention, a transgenic animal comprises, as a transgene a gene encoding a gene product that represses the transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. In another aspect of the invention, the transgene-encoded gene product induces the transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. For example, the transcription factor YY1 is a global repressor of cardiac hypertrophic responsive genes, while calcineurin generally induces the transcription of cardiac hypertrophic responsive genes.

Another aspect of the invention relates to cells isolated from the above-described transgenic animals. Preferably, such cells are derived from cardiac tissue and are cardiomyocytes. Cardiomyocytes isolated from the transgenic animals of the present invention can be used to test cardiotherapeutic properties of substances to which the cells are exposed *in vitro*. Thus, the cells are useful to screen for potential therapeutic agents

for treatment and/or prophylaxis of cardiac hypertrophy. Such cells can also be used to identify novel targets for prophylaxis and therapy of cardiac hypertrophy and cardiac hypertrophy-induced dysfunctions. These cells are also useful for investigative purposes, for examining signal transduction pathways involved in response to hypertrophic signals.

5           The invention further provides methods for screening substances for use in treating a cardiac hypertrophy-induced disease state (dysfunction).

          In some embodiments, enzyme-based screening assays are provided, wherein the enzymes used are involved in modulating levels of active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. The methods  
10           generally involve contacting the enzyme with a substance being tested and measuring an activity of the enzyme, compared to a control sample comprising the enzyme in the absence of the substance being tested

          In other embodiments, cell-based screening assays are provided, wherein substantially isolated cardiomyocytes are contacted with a substance to be tested, followed  
15           by measuring a cardiomyocyte function or parameter. An alteration in the cardiomyocyte function being measured, when compared to a control cell sample to which no substance is added, indicates that the substance is suitable for use in treatment of a cardiac hypertrophy-induced dysfunction. In some of these embodiments, the cardiomyocytes are derived from a transgenic animal of the invention. In other embodiments, the cardiomyocytes are  
20           derived from a non-transgenic animal.

          In still other embodiments, whole animal-based screening assays are provided. In these embodiments, invention encompasses the use of transgenic animals of the invention, or parts thereof, for testing cardiotherapeutic properties of substances administered to said animals, or for testing the activity of such substances in controlling or inhibiting the  
25           development of hypertrophic cardiomyopathy. Thus, according to another aspect of the invention, a method of screening and identifying or testing a drug or other substance for activity against the development of or in the treatment of hypertrophic cardiomyopathy, comprising treating a transgenic animal of the invention, or a part thereof, with said drug or other substance concerned and detecting or noting any reduced incidence in the  
30           development of hypertrophic cardiomyopathy and reduction in morbidity, as compared with corresponding animals that are not treated with the drug or substance, or detecting or noting an effectiveness in maintaining, restoring or improving heart function.

The invention further provides the use of compositions comprising substances identified by the methods of the present invention, as well as known substances, for treating cardiac hypertrophy and heart failure. Such compositions include substances which increase active levels of one or more gene products which normally repress  
5 transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, thereby reducing the expression of the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. Such compositions also include substances which reduce active levels of one or more gene products which normally increase transcription of at least one gene that is expressed in cardiomyocytes in  
10 response to a hypertrophic signal, thereby reducing the expression of the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. Further included are substances which are known modulators of gene products that modulate transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, for use in treating a cardiac hypertrophy-induced dysfunction. Included are known  
15 inhibitors of calcineurin, and derivatives of these inhibitors that inhibit a phosphatase activity of calcineurin.

The invention further provides methods of treating cardiac hypertrophy using modulators of gene products which modulate transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. In one aspect of the  
20 invention, calcineurin inhibitors are used to control cardiac hypertrophy and heart failure. The invention further provides methods of treating cardiac hypertrophy, or a cardiac hypertrophy-induced dysfunction, comprising administering to an individual in need thereof a substance which diminishes or reverses the progression of the dysfunction. In some embodiments, the substances inhibit expression of genes whose products modulate  
25 transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. In other embodiments, the substances inhibit an activity of a product of such a gene. In some embodiments, the methods comprise administering a composition comprising a known inhibitor of calcineurin, and derivatives of these inhibitors that inhibit a phosphatase activity of calcineurin.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents a model of nuclear signaling and transcriptional control of hypertrophy-induced embryonic gene expression. Abbreviations used are as follows: VDCC, voltage-dependent calcium channel; PLC, phospholipase C; AngII, angiotensin II; Et-1, endothelin-1; DAG, diacylglycerol; PKC, protein kinase C; IP3, inositol-3-phosphate; CAM, calmodulin.

Figure 2 shows inhibition of Angiotensin II- and phenylephrine-induced hypertrophy of primary cardiomyocytes by CsA and FK506. A-F, Primary rat cardiocytes in serum-free medium were stimulated with AngII (10 nM) or PE (10  $\mu$ M) for 72 hours. Cells were then fixed and stained with anti- $\alpha$ -actinin antibody to reveal sarcomeres and Hoechst stain to reveal nuclei. CsA (500 ng/ml) was added to one set of cultures at the time of AngII addition. G, Total RNA was isolated from primary cardiocyte cultures treated with AngII in the presence or absence of CsA as in A and analyzed for expression of GAPDH and ANF transcripts by dot blot. H, Primary rat cardiomyocytes were transiently transfected with a luciferase reporter gene linked to 3 tandem copies of the NF-AT consensus binding site. Cells were then treated with AngII or PE in the presence or absence of CsA, as described above. Forty-eight hours later, cells were harvested and luciferase activity was determined.

Figure 3 shows photographs of hearts of  $\alpha$ -MHC calcineurin transgenic mice. A, Control and  $\alpha$ -MHC-calcineurin transgenic littermates were sacrificed at 18 days of age and the hearts were removed and photographed. B,C, Hearts shown in A were sectioned longitudinally to reveal the ventricular chambers and interventricular septum. D,E, High magnification views of left ventricular walls of hearts shown in B and C, respectively. F, G, Transverse sections through hearts of control and calcineurin transgenics at 9 weeks of age stained with H&E. H, Sections from the calcineurin transgenic heart shown in G were stained with Masson trichrome to reveal collagen. ca, coronary artery; la, left atrium; lv, left ventricle; re, right atrium; rv, right ventricle. Bars in B,C,F,G,H = 1 mm. Bars in D,E = 50  $\mu$ m.

Figure 4 depicts the results of treatment of  $\alpha$ -MHC-calcineurin mice with CsA. A) The regimen for CsA treatment is shown. B)  $\alpha$ -MHC-calcineurin transgenic and nontransgenic mice, were treated with or without CsA (25 mg/kg), as indicated. Heart-to-body weight ratios are expressed  $\pm$  standard deviations. Transgenic littermates obtained

from male calcineurin transgenic #37 (see Table 1) were treated with CsA or vehicle alone beginning at 9 days of age, as described in Example 2. At 25 days of age, animals were sacrificed and hearts were removed and sectioned longitudinally. C) Hematoxylin and eosin-stained sections of hearts from nontransgenic (control) and transgenic mice treated with vehicle (center panel) or CsA (right panel). la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. The right atrium was removed from the control and both atria were removed from the transgenic treated with CsA. Bar = 2 mm.

Figure 5 shows photographs of hearts of  $\alpha$ -MHC CaMKIV transgenic mice. Control and  $\alpha$ -MHC-CaMKIV transgenic littermates were sacrificed at 3 weeks of age and the hearts were removed and sectioned longitudinally and stained with hematoxylin and eosin. la, left atrium; lv, left ventricle; re, right atrium; rv, right ventricle.

Figure 6 is a bar graph showing effects of mutated CaMKII and CaN on promoter-reporter constructs comprising ANF, skeletal  $\alpha$ -actin, and cardiac  $\alpha$ -actin promoters driving luciferase expression. Duplicate dishes of ventricular myocardial cells were co-transfected with reporter plasmids and CaMKII(1-290) or CaN<sub>mut</sub>, constitutively active forms of CaMKII and CaN, respectively. "Control" represents cells co-transfected with expression vectors without cDNA inserts and promoter-reporter plasmids. Values are corrected for transfection efficiency and reported as the mean  $\pm$  standard deviation (SD) from at least three experiments.

Figure 7 is a bar graph depicting effects of exogenous CaM kinase isoenzymes on ANF promoter-reporter activity. Duplicate dishes of ventricular cardiomyocytes were co-transfected with ANF promoter-reporter construct and expression constructs encoding various CaMK isoforms. "Control" represents cells co-transfected with expression vector without a cDNA insert and promoter-reporter plasmid. Values are corrected for transfection efficiency and reported as the mean  $\pm$  SD from at least three experiments.

Figure 8 is a bar graph depicting effects of PE, exogenous CaMKII or CaN on ANF nested deletion promoter-reporter constructs. Values are corrected for transfection efficiency and reported as the mean  $\pm$  SD from at least three experiments.

Figure 9 is a bar graph depicting stimulation of CaMKII activity by  $\beta$ 1-adrenergic receptor in neonate rat heart tissue. Neonatal (1-4 day old) heart tissue slices were isolated and treated with isoproterenol (ISO, 100  $\mu$ M), dolbutamine (DOB, 10  $\mu$ M), or vehicle alone (control). Propanolol (PROP, 20  $\mu$ M) was added 2 minutes before agonists were

added. After 30 seconds of treatment, autonomous CaMKII activity was assayed. Data are shown as percentage of the ratio of calcium independent activity divided by total activity and represented as the mean  $\pm$  SD from at least three experiments.

Figure 10 presents data showing the effects of adrenoceptor agonists on promoter-reporter activities for ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin, and a time course of effects of DOB on ANF message levels. Figure 10A, B, and C are bar graphs showing luciferase reporter levels after transfection of ANF, skeletal  $\alpha$ -actin, and cardiac  $\alpha$ -actin, respectively, into ventricular myocardial cells, and treatment, 24 hours after transfection, with PE (100  $\mu$ M), DOB (10  $\mu$ M), or TGF- $\beta$ 1 (1 ng/ml). Values are corrected for transfection efficiency and reported as the mean  $\pm$  SD from at least three experiments. Figure 10 D. Ventricular myocardial cells were switched to serum-free medium. DOB was added to the medium at various time points. "Control" represents cells treated with the DOB vehicle. 24 hours after incubation, total RNA was extracted and analyzed by Northern blot. Results are representative of n=3 or greater for each time point.

Figure 11 shows regulation of tTA-dependent target gene expression in primary rat cardiomyocytes. Cardiomyocytes were co-transfected with tTA expression plasmid pUHD15-1 and either a tTA-dependent luciferase construct pUHC13-3 or empty vector as control. 16 hours after transfection, cells were incubated in either medium without doxycycline (DOX) or medium with doxycycline. Figure 11A shows a time course of target gene transcription. Open circles, empty vector, no DOX; filled circles, empty vector, plus DOX; open triangles, pUHC13-3, no DOX; filled triangles, pUHC13-3, plus DOX. Figure 11B is a bar graph depicting the ability of tTA to stimulate transcription of the target gene and the inhibitory effect of doxycycline 12 hours after transfection.

Figure 12 is a graph showing dose dependency of the transcription of cardiac hypertrophy-sensitive genes in myocardial cells. 0.25  $\mu$ g tTA expression construct pUHD15-1, 0.25  $\mu$ g cardiac  $\alpha$ -actin promoter-reporter plasmid CardA-Luc, and various amounts of tTA-dependent CaN expression construct p10-3CaN were cotransfected into cells. Data are representative of six independent experiments. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 13 presents graphs showing a time course of transcription of cardiac hypertrophy-response genes induced by exogenous active CaN in myocardial cells. Data are normalized, and are expressed as a percentage of the maximum response. Data are

representative of three independent experiments. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 14 presents bar graphs depicting effects of activated CaN on transcription of hypertrophy-sensitive genes in myocardial cells. Data are representative of three independent experiments. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 15 is a graph depicting inhibition by CsA of CaN induction of cardiac hypertrophy response gene transcription. Myocardial cells were co-transfected with pUHD15-1, CardA-Luc, and either p10-3CaN or empty vector. Following transfection, cells were treated with 50 nM, 100 nM, or 150 nM CsA. At 24, 48, and 72 hours after treatment, luciferase activity was measured.

Figure 16 is a graph showing a time course of CsA inhibition of CaN induction of transcription of a cardiac hypertrophy-sensitive gene. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 17 is a bar graph depicting the effects of CsA treatment on transcriptional activation of cardiac hypertrophy-sensitive genes by CaN in myocardial cells. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 18 is a graph showing reversible regulation of transcriptional repression of cardiac hypertrophy-response gene expression by CsA. Values are reported as mean  $\pm$  SE; n=3 cultures.

Figure 19 is a graph showing time-dependent response of constitutively-active CaMKIV on skeletal  $\alpha$ -actin promoter-reporter activity in rat neonate cardiomyocytes.

Figure 20 is a graph showing dose-dependent response of constitutively active CaMKIV on cardiac  $\alpha$ -actin promoter-reporter activity in rat neonate cardiomyocytes.

Figure 21 is a graph showing dose-dependent response of constitutively active CaMKIV on skeletal  $\alpha$ -actin promoter-reporter activity in rat neonate cardiomyocytes.

Figure 22 is a graph showing dose-dependent response of constitutively active CaMKIV on ANF promoter-reporter activity in rat neonate cardiomyocytes.

### MODES FOR CARRYING OUT THE INVENTION

The present invention provides transgenic non-human animals which comprise, as transgenes, recombinant polynucleotides comprising a coding sequence encoding a gene product which modulates transcription of at least one gene that is expressed in

cardiomyocytes in response to a hypertrophic signal. Depending on the transgene, a transgenic animal of the invention has a substantially increased, or substantially reduced, probability of spontaneously developing cardiac hypertrophy.

The construction of transgenic animal models, using the recombinant polynucleotides for testing potential treatments for cardiac hypertrophy is described. The present invention provides transgenic non-human mammals comprising recombinant polynucleotides comprising a coding sequence encoding a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. The constructs are introduced into animal embryos using standard techniques such as microinjection or embryonic stem cells.

The present invention provides transgenic non-human animals and cells isolated from transgenic non-human animals, wherein the transgene encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, and wherein the isolated cells express the transgene.

The invention encompasses transgenic animals, or cells isolated therefrom, which can be used to screen for substances which can reduce cardiac hypertrophy, as measured by various parameters including, but not limited to, left ventricular mass/body weight, changes in cardiomyocyte size and organization, changes in cardiac gene expression and changes in cardiac function.

The present invention provides methods for detecting substances having therapeutic potential toward cardiac hypertrophy, using either transgenic animals or cells isolated therefrom. In addition, the present invention provides screening assays using substantially isolated enzymes involved in modulating levels of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. The screening assays can also be carried out using cells that are derived from non-transgenic animals.

The invention further provides methods for treating a cardiac hypertrophy-induced dysfunction, comprising administering to an individual in need of such treatment a composition comprising a substance which modulates active levels of at least one product of a gene expressed in response to a hypertrophic signal. The substances comprised in these compositions include those detected by the methods of the present invention, as well as known substances and their derivatives.



### General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); and *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991).

For techniques related to the production of transgenic animals, see, *inter alia*, Hogan et al (1986) *Manipulating the Mouse Embryo - A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986).

As used herein, the term "a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal" refers to any gene product, either a protein or an RNA, that modulates the transcription of at least one gene expressed in cardiomyocytes in response to a hypertrophic signal. It is also one which reduces or modulates activity of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal by reducing levels of transcription and/or translation or reducing protein activity e.g., by competitive or non-competitive inhibition, or by reducing protein concentration, and the like. The "modulation" can be either a down-regulation or an up-regulation of the gene expressed in cardiomyocytes in response to a hypertrophic signal.

A gene expressed in cardiomyocytes in response to a hypertrophic signal is also referred to herein as a "cardiac hypertrophy-sensitive gene" or a "cardiac hypertrophic-responsive gene". Examples of genes expressed in response to hypertrophic signals include, but are not limited to, atrial natriuretic factor (ANF),  $\alpha$ -skeletal actin, b-type natriuretic peptide (BNP), cardiac actin, and  $\beta$ -myosin heavy-chain ( $\beta$ -MHC), as shown schematically in Figure 1.

The term "transgenic animal" refers to a non-human animal, preferably a non-human mammal, which has integrated into its germline DNA one or more DNA sequences which have been introduced into the animal.

As used herein, the term "hypertrophic signal" indicates any stimulus, mechanical or chemical, which results in measurable symptoms of cardiac hypertrophy. Hypertrophic signals include, but are not limited to, mechanical stretch,  $\beta$ -adrenergic agonists,  $\alpha_1$ -adrenergic receptor agonists and angiotensin II. Symptoms of cardiac hypertrophy can be measured by various parameters including, but not limited to, left ventricular mass:body weight ratio; changes in cardiomyocyte size, mass, and organization; changes in cardiac gene expression; changes in cardiac function; fibroid deposition; changes in  $dP/dT$ , i.e., the rate of change of the ventricular pressure with respect to time; calcium ion flux; stroke length; and ventricular output.

The terms "transcriptional regulatory element" (TRE) and "transcriptional control region", "transcriptional response element", and "transcriptional control element", used interchangeably herein, refer to a polynucleotide sequence, preferably a DNA sequence, which selectively activates transcription of an operably linked polynucleotide sequence in a host cell. A TRE usually includes a promoter region and may optionally include, in addition to a promoter, other control sequences such as enhancers and silencers.

A "heterologous" TRE, promoter or enhancer is one that is not normally associated in a cell with, or is not derived from, a gene's 5' flanking sequence. In the context of a gene encoding a product that modulates active levels of at least one gene product of a hypertrophy-sensitive gene, examples of a heterologous promoter or enhancer include an  $\alpha$ -myosin heavy chain gene 5' flanking region.

The term "operably linked" relates to the orientation of polynucleotide elements in a functional relationship. A TRE is operably linked to a coding segment if the TRE promotes transcription of the coding sequence. Operably linked means that the DNA sequences being linked are generally contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by several kilobases, some polynucleotide elements may be operably linked but not contiguous.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or

deoxyribonucleotides. Thus, this term includes double- and single-stranded DNA and RNA. One skilled in the art would recognize that point mutations and deletions can be made to the promoter sequences and coding region sequences used in the present invention without altering the ability of the sequence to carry out its function, whether that function would be to activate transcription or to encode a functional protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A "substantially isolated" or "purified" polynucleotide is one that is substantially free of the materials with which it is associated in nature. By substantially free is meant at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of the materials with which it is associated in nature. As used herein, a "substantially isolated" polynucleotide also refers to recombinant polynucleotides, which, by virtue of origin or manipulation: (1) are not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) are linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

As used herein, the term "antibody" means an immunoglobulin molecule or a fragment of an immunoglobulin molecule having the ability to specifically bind to a particular antigen. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining antigen binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules of any isotype (IgA, IgG, IgE, IgD, IgM) but also the well-known active fragments F(ab')<sub>2</sub>, Fab, Fv, scFv, Fd, V<sub>H</sub> and V<sub>L</sub>. For antibody fragments, see, for example "Immunochemistry in Practice" (Johnstone and Thorpe, eds., 1996; Blackwell Science), p. 69.

As used herein, a "cardiac hypertrophy-induced dysfunction" is a condition or disease related to cardiac hypertrophy and includes cardiac hypertrophy, compound cardiac hypertrophy, dilated cardiac hypertrophy, decompensated cardiac hypertrophy, and heart failure. A cardiac hypertrophy-induced dysfunction is characterized by one or more

of the following symptoms or events: concentric enlarged ventricular mass; eccentric enlarged ventricular mass; progression toward dilated cardiac myopathy; extensive fibroid deposition; changes in dP/dT; cardiomyocyte disarray; calcium ion release and uptake (i.e.,  $\text{Ca}^{2+}$  flux); stroke shortening; diminished ventricular output; arrhythmias; tachycardia; changes in central demand; and heart failure.

### *Transgenic Animals*

The transgenic animals of the present invention include those which have a substantially decreased probability of spontaneously developing cardiac hypertrophy, and those which have a substantially increased probability of spontaneously developing cardiac hypertrophy, when compared with non-transgenic littermates. A "substantially increased" or a "substantially decreased" probability of spontaneously developing cardiac hypertrophy means that a statistically significant increase or decrease, respectively, of measurable symptoms of cardiac hypertrophy is observed when comparing the transgenic animal with a non-transgenic littermate(s).

The transgenic animals of the present invention are produced with transgenes which comprise a coding region that encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. Coding regions for use in constructing the transgenes of the present invention include, but are not limited to, those which encode MEF-2, p38, calcineurin (CaN), CaMKII $\alpha$ , CaMKIV, GATA4, and YY1. The coding regions may encode a complete polypeptide, or a fragment thereof, as long as the desired function of the polypeptide is retained, i.e., the polypeptide can modulate transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. The coding regions for use in constructing the transgenes of the present invention further include those containing mutations, including silent mutations, mutations which lead to a change in amino acid sequence but which do not result in functional alterations, mutations resulting in a more active protein, mutations that result in a constitutively active protein, mutations resulting in a protein with reduced activity, and dominant negative mutants. Coding regions can be derived from human, rat, or mouse sequences, several of which have been disclosed. Nucleotide sequences have been disclosed for mouse CaN catalytic subunit mRNA (GenBank Accession Nos. M81475 and J05479); human p38 mRNA (GenBank Accession No. AF015256); human YY1 mRNA (GenBank Accession No. Z14077); and

rat CaMKII $\alpha$  mRNA (GenBank Accession No. J02942). As noted above, these sequences, or variations or mutations thereof, can be used. As examples of constitutively active mutants, constitutively active forms of CaN, CaMKII and CaMKIV have been described. Sun et al. (1994) *Genes Dev.* 8:2527-2539; Cruzalegui and Means (1993) *J. Biol. Chem.* 268:26171-26178; and O'Keefe et al. (1992) *Nature* 357:692-694.

The transgenes, for use in generating transgenic animals, which comprise a coding region that encodes a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, include those in which the gene product which modulates transcription of at least one hypertrophy-sensitive gene is 1) under the control of a cardiomyocyte-specific promoter; 2) constitutively expressed in cardiomyocytes under the control of a cardiomyocyte-specific promoter; 3) under the control of a cardiomyocyte-specific promoter but is expressed in an inducible manner.

#### ***Transcriptional regulatory elements***

Transgenic animals of the present invention comprise, as a transgene, a polynucleotide comprising a coding region encoding a gene product that modulates expression of at least one gene expressed in cardiomyocytes in response to a hypertrophic signal. Transcription of the coding region is controlled by a transcriptional regulatory element (TRE). TREs suitable for use in the present invention include those which direct the transcription of a coding region to which they are operably linked preferentially in cardiomyocytes. By "preferentially" is meant that the expression of the transgene in cardiomyocytes is at least about 10-fold, more preferably at least about 10-fold to about 50-fold, even more preferably at least about 50-fold to 100-fold, even more preferably more than 100-fold greater than that in non-cardiomyocytes. Preferably, expression of the transgene is below detectable limits in cells other than cardiomyocytes, as indicated by reporter gene assays, such as those described in the Examples.

A variety of cardiomyocyte-specific TREs have been described in the literature and can be used in the present invention. These include, but are not limited to, TREs derived from the following genes: myosin light chain-2,  $\alpha$ -myosin heavy chain, AE3, cardiac troponin C, and cardiac  $\alpha$  actin. Franz et al. (1997) *Cardiovasc. Res.* 35:560-566; Robbins et al. (1995) *Ann. N.Y. Acad. Sci.* 752:492-505; Linn et al. (1995) *Circ. Res.* 76:584-591; Parmacek et al. (1994) *Mol. Cell. Biol.* 14:1870-1885; Hunter et al. (1993) *Hypertension* 22:608-617; and Sartorelli et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4047-4051.

In one embodiment, the TRE comprises a promoter region from the 5' flanking region of an  $\alpha$ -MHC gene. A 5443 base 5' flanking sequence for the mouse  $\alpha$ -MHC gene is provided in GenBank under accession number U71441. Although the entire 5.4 kb sequence can be used in the transgenes of the present invention, portions thereof which  
5 direct transcription of an operably linked coding region preferentially in cardiomyocytes can also be used. The  $\alpha$ -MHC expression vector described in Example \* can be used to insert a desired coding region such that the coding region will be operably linked to the  $\alpha$ -MHC promoter. Jones et al. (1994) *Dev. Dyn.* 22:117-128.

In another embodiment, the TRE comprises a promoter region from the 5' flanking  
10 region of a b-type natriuretic peptide gene (BNP). Thuerlauf and Glembotski (1997) *J. Biol. Chem.* 272:7464-7472; and LaPointe et al. (1996) *Hypertension* 27:715-722. A nucleotide sequence for a 5' flanking region of human BNP has been disclosed (GenBank Accession No. D16641).

In other embodiments, the coding region is operably linked to an inducible  
15 regulatory element(s). A variety of inducible promoter systems have been described in the literature and can be used in the present invention. These include, but are not limited to, tetracycline-regulatable systems, (WO 94/29442; WO 96/40892; and WO 96/01313); hormone-responsive systems, interferon-inducible systems, metal-inducible systems, and heat-inducible systems, (WO 93/20218); and ecdysone-inducible systems. Some of these  
20 systems, including ecdysone-inducible and tetracycline-inducible systems are commercially available from Invitrogen (Carlsbad, CA) and Clontech (Palo Alto, CA), respectively.

In some embodiments, the inducible (regulatable) promoter system comprises a tetracycline-binding operator, *tetO*, regulated by addition of tetracycline (or an analog  
25 thereof) to the animal's water or diet, operably linked to a coding region whose product modulates transcription of at least one gene expressed in cardiomyocytes in response to a hypertrophic signal. Systems suitable for use in the present invention are well known. Yu et al. (1996) *Circ. Res.* 79:691-697; Gossen et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; and Hillen and Wissmann, in *Protein-Nucleic Acid Interaction, Topics in*  
30 *Molecular and Structural Biology*, Saenger and Heinemann, eds. Macmillan, London (1989), pp. 143-162. In general, this system makes use of a hybrid fusion protein, the tetracycline transactivator (tTA), which comprises a DNA binding domain, capable of

binding *tetO*, derived from a polypeptide such as the tet repressor (*tetR*) protein from *E. coli*, coupled to a transcriptional activation domain such as from the viral protein VP16, such that when tTA binds to a minimal promoter containing *tetO* sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation, presumably by causing a conformational change in the *tetR* portion of tTA, blocking binding of tTA to *tetO*. Gene activation occurs by removing tetracycline.

In addition, cell type-specific TREs can be used. Cell type-specific TREs include, but are not limited to, those derived from the following exemplary genes (cell type in which the TREs are specifically functional are in parentheses): vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetase I (small intestine), Na-K-Cl transporter (kidney), prostate specific antigen (prostate), and glandular kallikrein-1 gene (prostate).

Another class of TREs are those which activate transcription of an operably linked polynucleotide in response to hypoxic conditions. These include TREs regulated, at least in part, by hypoxia-inducible factor-1. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemain and Krasnow (1997) *Cell* 89:9-12. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500; and Jiang et al. (1997) *Cancer Res.* 57:5328-5335.

A wide variety of viral promoters are known in the art and can be used, including, but not limited to, a cytomegalovirus (CMV) promoter, a Rous Sarcoma Virus (RSV) promoter, an SV40 promoter, and an MMTV LTR promoter. Depending on the nature of their regulation, promoters may be constitutive or regulatable by experimental conditions.

Other regulatory sequences which can be included in a transgene include polyadenylation signals, and termination signals, the latter which can serve to enhance message levels and to minimize read-through into other sequences. These regulatory sequences are known in the art.

#### ***Polynucleotides for use in making transgenic animals***

Substantially isolated polynucleotides for use in making a transgenic animal of the present invention include polynucleotides comprising a coding region encoding a gene product which modulates transcription of at least one gene that is expressed in

cardiomyocytes in response to a hypertrophic signal. Such gene products include polypeptides, antisense polynucleotides, and ribozymes.

Polypeptide gene products which modulate transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal include, but are not limited to, calcineurin, YY1, p38/Hog1, CaMKIV, and CaMKII $\alpha$ .

Several cDNA clones suitable for use in the present invention for creating transgenic animals have been described: p38, Wang et al. (1997) *J. Biol. Chem.* 272:23668-23674 (human sequence, GenBank Accession No. AF015256); calcineurin catalytic subunit, Muramatsu and Kincaid (1992) *Biochim. Biophys Res. Comm.* 188:265-271 (human sequence for catalytic subunit, GenBank Accession No. S46622), Kincaid et al. (1990) *J. Biol. Chem.* 265:11312-11319 (mouse sequences for catalytic subunit, GenBank Accession Nos. J05479 and M81475); and YY1, Shi et al. (1991) *Cell* 67:377-388 (human sequence, GenBank Accession No. Z14077).

Expression clones for making transgenic mice can also be constructed such that the coding region bears one or more mutations that affect the activity of the encoded protein. For example, a CaM mutant lacking amino acids 75-82 binds Ca<sup>2+</sup> with kinetic properties similar to those of wild-type CaM, but is unable to activate several CaM-dependent target enzymes *in vitro*. Van Berkum et al. (1990) *J. Biol. Chem.* 265:3750-3756.

Useful mutations include those that result in constitutively active forms of the encoded protein. For example, genes encoding constitutively active mutant forms of CaMKIV, CaMKII $\alpha$ , and CaN can be used to generate transgenic animals. For example, a construct termed pSR-CaMKII(1-290), which harbors a constitutively-active truncated mutant form of this wild-type CaMKII has been described. Sei et al. (1991) *J. Biol. Chem.* 266:15910-15916.

Other useful mutations include dominant negative mutants. For example, a dominant negative mutant of a calcium/calmodulin-dependent kinase can be one that is truncated or otherwise mutated so that it loses its calcium ion sensitivity, but retains sensitivity to calmodulin. It can also be a mutant that has lost kinase activity, but still binds calmodulin. Since such a mutant would bind CaM, but would lack enzymatic activity, downstream events would be affected.

Polynucleotide gene products which are useful include antisense polynucleotides, ribozymes, and triple helix polynucleotides that modulate the expression of a product of at



least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. Antisense polynucleotides and ribozymes have been amply described. S. T. Crooke and B. Lebleu, eds. *Antisense Research and Applications* (1993) CRC Press; and *Antisense RNA and DNA* (1988) D.A. Melton, Ed. Cold Spring Harbor Laboratory Cold Spring Harbor, NY. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. An example of an antisense polynucleotide is an oligodeoxyribonucleotide derived from the translation initiation site, e.g., between -10 and +10 regions of the relevant nucleotide sequence.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding protein complex components.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays. See, Draper PCT WO 93/23569; and U.S. Pat. No. 5,093,246.

Nucleic acid molecules used in triple helix formation for the inhibition of transcription are generally single stranded and composed of deoxyribonucleotides. The base composition must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC<sup>+</sup> triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example,

containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

5           Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

10           Anti-sense RNA and DNA molecules, ribozymes, and triple helix molecules can be prepared by any method known in the art for the synthesis of DNA and RNA molecules. See, Draper, id. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art including, but not limited to, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by  
15           *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably or  
20           transiently into cells.

          Various well-known modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of  
25           phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

          Gene products which modulate transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal can be identified by any means known in the art, including, but not limited to, a yeast two-hybrid system, a variety of  
30           which are known in the art and described in more detail below. A transcription activation assay such as the yeast two-hybrid system allows for the identification and manipulation of protein-protein interactions. Fields and Song (1989) *Nature* 340:245-246. The conceptual

basis for a transcription activation assay is predicated on the modular nature of transcription factors, which consist of functionally separable DNA-binding and trans-activation domains. When expressed as separate proteins, these two domains fail to mediate gene transcription. However, the ability to activate transcription can be restored if the DNA-binding domain and the trans-activation domain are bridged together through a protein-protein interaction. These domains can be bridged, for example, by expressing the DNA-binding domain and trans-activation domain as fusion proteins (hybrids), where the proteins that are appended to these domains can interact with each other. The protein-protein interaction of the hybrids can bring the DNA-binding and trans-activation domains together to create a transcriptionally competent complex. A known component of a signal transduction pathway such as GATA4 can act as the "bait", and can be fused to a DNA-binding protein. Expression cDNA libraries can be made which allow the expression of fusion proteins comprising a protein fused to a transcriptional activator. Proteins which bind functionally to GATA4 then allow transcription of the *lacZ* gene to take place. The product of the *lacZ* gene can then be detected by adding a chromogenic substrate to growth substrate. Kits for conducting such assays are commercially available.

#### ***Nucleic Acid Constructs Used to Make Transgenic Animals***

Constructs for use in generating transgenic animals comprise a TRE for expression of the construct in an animal cell and a region encoding a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

In some embodiments, a construct for use in generating a transgenic animal comprises a region encoding a gene product which modulates transcription of at least one hypertrophy-sensitive gene in cardiomyocytes, wherein the region encoding the gene product is operably linked to a heterologous promoter such that the gene product is over-expressed in cardiomyocytes. "Over-expressed" indicates that the gene product is expressed at least about 2-fold to about 5-fold, preferably at least about 5-fold to about 50-fold, more preferably at least about 50-fold to about 100-fold, even more preferably at least about 100-fold, when compared with expression of the gene in wild-type cells or when compared with expression of the gene operably linked to its homologous promoter.

In other embodiments, the construct for use in generating a transgenic animal comprises a region encoding a gene product which modulates transcription of at least one

hypertrophy-sensitive gene in cardiomyocytes, wherein the region encoding the gene product is operably linked to a heterologous TRE and the region encoding the gene product is mutated such that the encoded gene product is constitutively active in cardiomyocytes.

5           In other embodiments, the construct for use in generating a transgenic animal comprises a region encoding a gene product which modulates transcription of at least one hypertrophy-sensitive gene in cardiomyocytes, wherein the region encoding the gene product is operably linked to a heterologous TRE and wherein transcription of the region encoding the gene product is inducible. Inducible systems are known in the art and are  
10           described herein.

          In other embodiments, the construct for use in generating a transgenic animal comprises a region encoding a gene product which modulates transcription of at least one hypertrophy-sensitive gene in cardiomyocytes, wherein the gene product is a dominant negative mutant.

15           DNA clones for microinjection can be prepared by any means known in the art. For example, DNA clones for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed using standard techniques. The DNA bands are visualized by staining with ethidium bromide, and the band containing the expression sequences is excised. The excised band is then  
20           placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a 1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elute-D™ column. The  
25           column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and  
30           precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 µg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA.

          Other methods for purification of DNA for microinjection are described in Hogan et al. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring

Harbor, NY, 1986), in Palmiter et al. *Nature* 300:611 (1982); in *The Qiagenologist, Application Protocols*, 3rd edition, published by Qiagen, Inc., Chatsworth, CA.; and in Sambrook et al. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

5      ***Testing Transgene Constructs in Cell Culture***

The transgene constructs can be tested, if desired, for activity in *in vitro* culture, as described in the Examples. TRE activity can be tested, for example, by introducing into cultured cardiomyocytes a polynucleotide construct comprising a TRE operably linked to a gene whose ability to modulate the transcription of at least one gene that is expressed in  
10      cardiomyocytes in response to a hypertrophic signal is to be tested, and monitoring the expression of the hypertrophy-sensitive gene or genes. Alternatively, the activity of a TRE can be tested by introducing into cultured cardiomyocytes a polynucleotide construct comprising a TRE operably linked to a reporter gene which provides for a detectable, desirably quantifiable, signal.

15      The sequence(s) to be tested can be inserted into a vector such that they are operably linked to an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the *lacZ* gene), luciferase, (encoded by the *luc* gene), alkaline phosphatase, green fluorescent protein, and horse radish peroxidase. Such vectors and assays are readily available, from,  
20      *inter alia*, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran.

25      The construct can be prepared in accordance with conventional ways, introducing each of the components of the construct into a plasmid by employing convenient restriction sites, PCR to introduce specific sequences at the termini, which may include restriction sites, and the like. After the expression construct has been prepared, it can be introduced into the cells by any convenient means.

30      Methods for introducing the expression construct into cells include transfection, complexing with cationic compounds, lipofection, electroporation, and the like. An expression construct can be introduced into a cell in association with any of the delivery vehicles, including viral and non-viral delivery vehicles, as described below. Following

introduction of the expression construct into the cells, expression of the reporter gene can be determined by conventional means. Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags, radioimmunoassays or other immunological assays. Transfection efficiency can be monitored by co-transfecting an expression construct comprising a constitutively active promoter operably linked to a reporter gene.

The activity of a TRE in a TRE-reporter gene construct can be assessed after transient expression, or stable cell lines can be created. If a stable cell line is to be created, then the construct also contains a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media. Alternatively, the selectable marker can be contained on a construct separately from the construct containing the TRE-reporter gene, and the two constructs introduced simultaneously into host cells.

To assess the ability of one or more gene products to modulate the activity of a TRE, an expression construct comprising a gene encoding a product to be tested can be co-transfected into cardiomyocytes along with the TRE-reporter gene construct. The effect of the gene product on the expression of the reporter gene can then be assessed as described above.

To assess the ability of one or more gene products to modulate the transcription of one or more hypertrophy-sensitive genes, an expression construct comprising a TRE from a hypertrophy-sensitive gene can be operably linked to a reporter gene as described above, and introduced into cells as described above. Expression constructs comprising a gene encoding a product whose ability to modulate transcription of the one or more hypertrophy-sensitive genes is to be tested can be co-transfected into the cells. The effect of the gene product on the expression of the reporter gene can then be assessed as described above.

Optionally, additional agents can be added to the culture medium such as receptor agonist and antagonists, including, but not limited to, adrenergic receptor agonists or antagonists. The effect, if any, on transcription of a hypertrophy-sensitive gene can be measured as described above.

5 Agonists and antagonists suitable for use in the present invention include, but are not limited to,  $\beta$ 1-specific agonists such as albuterol (ALB) and dobutamine (DOB);  $\beta$ 1-specific antagonists such as metoprolol (MET);  $\alpha$ 1-specific agonists such as phenylephrine (PE);  $\alpha$ 1-specific antagonists such as prazosin; mixed  $\beta$  agonists such as isoproterenol (ISO); mixed  $\beta$  antagonists such as propranolol (PROP); and Angiotensin II.

#### 10 ***Delivery of polynucleotide constructs into cells***

A polynucleotide construct can be introduced into a cell in a variety of ways. Delivery vehicles suitable for delivery of a polynucleotide into a cell (whether *in vivo*, *ex vivo*, or *in vitro*) include viral and non-viral delivery vehicles. Generally, a polynucleotide sequence will be operably linked to a transcriptional regulatory element (TRE) and a  
15 heterologous polynucleotide. A polynucleotide comprising a coding region encoding a gene product which modulates transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, or a polynucleotide comprising a hypertrophy-sensitive gene can be contained within a cloning or expression vector, using methods well known in the art. These vectors (especially expression vectors) can in turn  
20 be manipulated to assume any of a number of forms which may, for example, facilitate delivery to and/or entry into a cell. Delivery of the polynucleotide constructs of the invention to eukaryotic cells, particularly to mammalian cells, more particularly to cardiomyocytes, can be accomplished by any suitable art-known method. Delivery can be accomplished *in vivo*, *ex vivo*, or *in vitro*.

25 Delivery vehicles suitable for incorporation of a polynucleotide for introduction into a host cell include non-viral vehicles and viral vectors. Verma and Somia (1997) *Nature* 389:239-242.

A wide variety of non-viral vehicles for delivery of a polynucleotide are known in the art and are encompassed in the present invention. A polynucleotide can be delivered to  
30 a cell as naked DNA (U.S. Patent No. 5,692,622; WO 97/40163). Alternatively, a polynucleotide can be delivered to a cell associated in a variety of ways with a variety of

substances (forms of delivery) including, but not limited to cationic lipids; biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria. A delivery vehicle can be a microparticle. Mixtures or conjugates of these various substances can also be used as delivery vehicles. A polynucleotide can be associated non-covalently or covalently with these various forms of delivery. Liposomes can be targeted to a particular cell type, e.g., to a cardiomyocyte.

Viral vectors include, but are not limited to, DNA viral vectors such as those based on adenoviruses, herpes simplex virus, poxviruses such as vaccinia virus, and parvoviruses, including adeno-associated virus; and RNA viral vectors, including, but not limited to, the retroviral vectors. Retroviral vectors include murine leukemia virus, and lentiviruses such as human immunodeficiency virus. Naldini et al. (1996) *Science* 272:263-267.

Non-viral delivery vehicles comprising a polynucleotide can be introduced into host cells and/or target cells by any method known in the art, such as transfection by the calcium phosphate coprecipitation technique; electroporation; electroporomeabilization; liposome-mediated transfection; ballistic transfection; biolistic processes including microparticle bombardment, jet injection, and needle and syringe injection; or by microinjection. Numerous methods of transfection are known to the skilled worker in the field.

Viral delivery vehicles can be introduced into cells by infection. Alternatively, viral vehicles can be incorporated into any of the non-viral delivery vehicles described above for delivery into cells. For example, viral vectors can be mixed with cationic lipids (Hodgson and Solaiman (1996) *Nature Biotechnol.* 14:339-342); or lamellar liposomes (Wilson et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:3471; and Faller et al. (1984) *J. Virol.* 49:269).

For *in vivo* delivery, the delivery vehicle(s) can be introduced into an individual by any of a number of methods, each of which is familiar in the art.

#### ***Animal Sources***

Animals which are suitable for the generation of transgenic animals of the present invention are non-human animals, preferably mammals, and include, but are not limited to, mice, rats, rabbits, pigs, cows and sheep.



Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), and Harlan Sprague Dawley (Indianapolis, IN). For example, if the transgenic animal is a mouse, many mouse strains are suitable, but C57BL/6 female mice are preferred for embryo retrieval and transfer. C57BL/6 males can be used for mating and vasectomized C57BL/6 studs can be used to stimulate pseudopregnancy. Vasectomized mice and rats can be obtained from the supplier.

### *Transgenic animals*

Transgenic animals can be made by any known procedure, including microinjection methods, and embryonic stem cells methods. The procedures for manipulation of the rodent embryo and for microinjection of DNA are described in detail in Hogan *et al.*, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), the teachings of which are generally known and are incorporated herein.

As an example of microinjection, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO<sub>2</sub> asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5 % BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 5% CO<sub>2</sub>, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Randomly cycling adult female mice are paired with vasectomized males. C57BL/6 or Swiss mice or other comparable strains can be used for this purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5 % avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are

placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

### ***Identification and Characterization of Transgenic Animals***

Transgenic animals can be identified by analyzing their DNA. For this purpose, when the transgenic animal is a rodent, tail samples (1 to 2 cm) can be removed from three week old animals. DNA from these or other samples can then be prepared and analyzed by Southern blot, PCR, or slot blot to detect transgenic founder (F<sub>0</sub>) animals and their progeny (F<sub>1</sub> and F<sub>2</sub>).

#### **1. Pathological studies**

The various F<sub>0</sub>, F<sub>1</sub> and F<sub>2</sub> animals that carry a transgene can be analyzed by any of a variety of techniques, including immunohistology, electron microscopy, electrocardiography and making determinations of total and regional heart weights, measuring cardiomyocyte cross-sectional areas and determining numbers of cardiomyocytes. Immunohistological analysis for the expression of a transgene by using an antibody of appropriate specificity can be performed using known methods. Morphometric analyses to determine regional weights, cardiomyocyte cross-sectional areas and numbers of cardiomyocyte nuclei can be performed using known methods. Hearts can be analyzed for function, histology and expression of fetal cardiac genes.

#### **2. Analysis of Transgene Expression by Measuring mRNA Levels**

Messenger RNA can be isolated by any method known in the art, including, but not limited to, the acid guanidinium thiocyanate-phenol:chloroform extraction method (Chomczynski and Sacchi (1987) *Anal. Biochem.* 162:156-159), from cell lines and tissues of transgenic animals to determine expression levels by Northern blots, RNase, quantitative PCR, and nuclease protection assays.

#### **3. Analysis of Transgene Expression by Measuring Protein Levels**

Protein levels can be measured by any means known in the art, including, but not limited to, western blot analysis, ELISA and radioimmunoassay, using one or more antibodies specific for the protein encoded by the transgene.

For Western blot analysis, protein fractions can be isolated from tissue homogenates and cell lysates and subjected to Western blot analysis as described by, for

example, Harlow *et al. Antibodies: A Laboratory Manual*, (Cold Spring Harbor, NY, 1988).

For example, the protein fractions can be denatured in Laemmli sample buffer and electrophoresed on SDS-Polyacrylamide gels. The proteins are then transferred to nitrocellulose filters by electroblotting. The filters are blocked, incubated with primary antibodies, and finally reacted with enzyme conjugated secondary antibodies. Subsequent incubation with the appropriate chromogenic substrate reveals the position of the transgene-encoded proteins.

### ***Crossing Transgenic Animals***

The present invention further provides transgenic non-human animals that are progeny of crosses between a transgenic animal of the invention and a second animal. The transgenic animals of the present invention can be bred with other animals to identify other factors that influence the hypertrophic response and which could also serve as targets for drugs. Transgenic animals can be bred with other transgenic animals, where the two transgenic animals were generated using different transgenes, to test the effect of one gene product on another gene product or to test the combined effects of two gene products.

In some embodiments, a first transgenic animal comprising as a transgene a polynucleotide encoding a gene product which increases transcription of at least one cardiac hypertrophy-sensitive gene operably linked to a heterologous, cardiomyocyte-specific TRE, wherein expression of the transgene is embryonic lethal, or otherwise deleterious such that the animals do not survive long enough for analysis, the transgene can be placed under transcriptional control of an inducible promoter, such as the tet inducible system described above, such that the transgene is substantially not expressed until desired. Such animals can be crossed with a tTA constitutive "founder" transgenic animal, which comprises as a transgene a polynucleotide comprising a tTA under tetracycline control.

In other embodiments, a first transgenic animal, comprising as a transgene a polynucleotide encoding a gene product that increases transcription of at least one cardiac hypertrophy-sensitive gene operably linked to a heterologous, cardiomyocyte-specific TRE, such that the first animal displays one or more symptoms of cardiac hypertrophy, is crossed with a second transgenic animal comprising as a transgene a dominant negative mutant of a gene product which increases transcription of at least one cardiac hypertrophy-

sensitive gene. The progeny of such a cross would display a reduction of one or more symptoms associated with cardiac hypertrophy.

In other embodiments, a first transgenic animal, comprising as a transgene a polynucleotide encoding a gene product which decreases or represses transcription of at least one cardiac hypertrophy-sensitive gene operably linked to a heterologous, cardiomyocyte-specific TRE, such that the first animal does not display a symptom of cardiac hypertrophy and is substantially resistant to developing cardiac hypertrophy, is crossed with a second transgenic animal comprising as a transgene a polynucleotide encoding a constitutively active mutation of a gene product which increases transcription of at least one cardiac hypertrophy-sensitive gene.

In other embodiments, a first transgenic animal, comprising as a transgene a polynucleotide which is an antisense polynucleotide targeted to a gene product which modulates transcription of at least one cardiac hypertrophy-sensitive gene, is crossed with a second transgenic animal comprising as a transgene a polynucleotide encoding a constitutively active mutation of a gene product which increases transcription of at least one cardiac hypertrophy-sensitive gene, wherein the antisense polynucleotide inhibits expression of the transgene of the second transgenic animal.

#### ***Screening of Substances for Treatment of Cardiac Hypertrophy***

The transgenic animals, animal cells isolated from transgenic animals, or isolated enzymes can be used to screen substances for a potential effect in the treatment of cardiac hypertrophy.

##### **Enzyme-based screening assays**

In enzyme-based screening assays, substantially isolated enzymes involved in response to a hypertrophic signal in cardiomyocytes are contacted with a substance to be tested. Activity of the enzyme is measured in the presence of the substance, after a suitable time. Conditions and times sufficient for interaction of an enzyme agonist or antagonist candidate with the enzyme will vary with the enzyme, however, conditions generally suitable for binding occur between about 4°C and about 40°C, preferably between about 4°C and about 37°C; in a buffered solution containing appropriate ions in an appropriate concentration range, which may vary for a given enzyme; and within a pH range of between 5 and 9. Sufficient time for the binding and response will generally be between about 1 millisecond and about 24 hours after exposure.

Enzyme activity in the presence of the substance being tested is compared with the activity in the absence of the substance being tested. An increase or decrease in the activity being measured is an indication that the substance is suitable for use in reducing a cardiac hypertrophy-induced dysfunction. A substance is considered to have an effect on enzyme activity if the activity being measured is increased or decreased at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold or more, relative to the activity of the enzyme activity measured in the absence of the substance being tested. The substance can be further tested in a cell based and/or whole animal-based assay, as described below.

Enzymes which are involved in a response to a hypertrophic signal in cardiomyocytes include, but are not limited to, calcineurin, CaMKIV, CaMKII $\alpha$ , p38, YY1, a CaMK kinase, and MKK6. Enzymes can be used in their native form; in mutated forms having altered enzymatic activities and/or *in vitro* stability or solubility; in mutant forms in which a regulatory or other component of the enzyme is absent, but the enzyme retains an enzymatic activity related to response to a hypertrophic signal; covalently or non-covalently associated with another molecule to confer *in vitro* stability, to facilitate attachment to a solid support, to provide an antigenic determinant, and the like. The enzymes can be conjugated to a label capable of producing a detectable signal or other functional moieties. Suitable labels include, but are not limited to, radionuclides, other enzymes, substrates, cofactors, inhibitors, fluorescent dyes, chemiluminescent dyes, bioluminescent compounds and magnetic particles.

Preferably, for use in a screening assay, an enzyme is substantially purified. A "substantially isolated" or "purified" enzyme is one that is substantially free of the materials with which it is associated in nature, particularly of other proteinaceous material or substances which may inhibit an enzymatic activity related to a response to a hypertrophic signal. By substantially free is meant at least 50%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90% free of the materials with which it is associated in nature. As used herein, a "substantially isolated" enzyme also refers to recombinant enzymes, which, by virtue of origin or manipulation: (1) are not associated with all or a portion of an enzyme with which it is associated in nature, (2) are linked to a polypeptide other than that to which it is linked in nature, or (3) does not occur in nature.

Enzymes for use in these assays are obtainable by any method known in the art, including, but not limited to, natural sources, chemical synthesis or recombinant techniques. An enzyme for use in the screening assays of the invention can be purified by any known method. A wide variety of methods are known in the art. See, for example, 5 *Protein Purification: Principles and Practice*, R. Scopes, ed. (1987) Springer-Verlag.

Enzyme activity can be determined by any known method. For example, kinase activity of p38 can be measured by incubating the enzyme together with a protein substrate and  $\gamma^{32}\text{P}$ -ATP, and detecting  $^{32}\text{P}$  incorporation into the substrate, for example, by scintillation counting. Assays for p38 kinase activity have been described. See, for 10 example, PCT Publication No. WO 98/27098.

#### Cell-based screening assays

In the cell-based screening assays of the present invention, the compound to be tested is introduced into the culture media of cells, over period of time and in various dosages, then the cells are examined for one or more functions, including, but not limited 15 to, changes in expression levels of a hypertrophy-sensitive gene, changes in levels of a polypeptide product of a hypertrophy-sensitive gene, and changes in cell size and/or morphology.

Screening assays for determining the therapeutic potential of compounds can be performed using animal cells derived from transgenics for the disclosed transgene 20 constructs in cell cultures and stably or transiently transfected with the disclosed constructs. Cell-based screening assays can also be performed using primary cells, such as cardiomyocytes, derived from a non-transgenic animal, the cells being stably or transiently transfected with the polynucleotide constructs, or being stimulated with hypertrophic signals such as Angiotensin II or  $\alpha$ -adrenergic agonists.

25 To detect a change in levels of a polypeptide product of a hypertrophy-sensitive gene, a variety of assay methods can be used, each of which is known in the art. For example, an immunological assay such as an ELISA can be used, employing one or more antibodies specific for one or more polypeptide products of a hypertrophy-sensitive gene.

To detect a change in expression levels of a hypertrophy-sensitive gene, any known 30 method can be used, including, but not limited to, hybridization with a polynucleotide probe which is complementary to an RNA product of the gene; and a polymerase chain reaction using oligonucleotide primers that prime extension of a cDNA copy of an RNA

product of the gene. Nucleotide sequences of some hypertrophy-sensitive genes have been disclosed, and this information can be used to design oligonucleotide primers.

Alternatively, the cells can be transfected with a reporter gene construct comprising a reporter gene operably linked to a TRE, particularly one derived from a hypertrophy-sensitive gene. In these assays, the readout from these assays is level of active reporter gene product. Nucleotide sequences of 5' flanking regions of some hypertrophy-sensitive gene have been disclosed, and can be used to generate constructs comprising a cardiac hypertrophy-sensitive gene TRE operably linked to a reporter gene. Such nucleotide sequences include, for example, rat ANF 5' flanking sequences (GenBank Accession No. J03267); and mouse  $\beta$ -MHC 5' flanking sequences (GenBank Accession No. U86076).

The cell-based screening assays described herein can be used as primary screens, or alternatively, as secondary screens to further test compounds which are active in an above-described enzyme-based screen.

For these assays, suitable cells include, but are not limited to, primary cardiomyocytes, isolated from either transgenic or non-transgenic animals. Other cells suitable for use in cell-based screening assays include non-cardiomyocyte cell types such as fibroblasts.

In some embodiments, cells are transfected with a construct comprising a TRE from a hypertrophy-responsive gene operably linked to a reporter gene. Reporter genes are known in the art and described above. One example is a green fluorescent protein (GFP) and variants thereof. See, for example, Heim and Tsien (1996) *Curr. Biol.* 6:178-182; Mitra et al. (1996) *Gene* 173:13-17; and Yang et al. (1996) *Nucl. Acids Res.* 24:4592-4593. The transfected cells are then placed in 96-well culture plates at  $1.5$  to  $2.5 \times 10^4$  cells per well in a suitable culture medium, such as Dulbecco's minimal essential medium plus 10% fetal bovine serum. Following overnight incubation at  $37^\circ\text{C}$  in an incubator equilibrated with 5% carbon dioxide, media are removed and replaced with media containing a compound to be tested and incubated for a suitable period of time. For the present invention, a suitable time period for incubation with a substance to be tested can be from about 0.5 hours to about 24 hours. In addition to the test compound, a hypertrophy-inducing substance is added to the culture medium. The hypertrophy-inducing substance can be added simultaneously with, before, or after, addition of the test substance. Hypertrophy-inducing substances include, but are not limited to, angiotensin II and PE.

Stocks containing the compound to be tested are first prepared in suitable solvent. Several dilutions of the test compound are tested. Appropriate controls include cells to which solvent containing no test substance is added, and cells to which a test substance was added, but to which no hypertrophy-inducing substance is added.

5           After treatment, an effect of the test compound is measured using a suitable assay for the reporter gene product.

Reporter genes which may be employed are well known and include, but are not limited to, luciferase; a green fluorescent protein (GFP), for example, a GFP from *Aequorea victoria*, or any of a variety of GFP known in the art;  $\beta$ -galactosidase,  
10 chloramphenicol acetyl transferase; immunologically detectable protein "tags" such as human growth hormone; and the like. See, for example, Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987) and periodic updates.

Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter  
15 gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags, radioimmunoassays or other immunological assays.

A substance is considered to have an effect if transcription of the operably linked reporter gene is increased or decreased in cells at least about 2-fold, more preferably at  
20 least about 5-fold, more preferably at least about 10-fold or more, relative to control cells which were not exposed to the substance.

Cytotoxic effects of the substances can be measured by any means known in the art. For example, cytotoxic effects of the substances can be measured by a modification of the method of Hansen et al. (1989) *J. Immunol. Method.* 119:203-210. To the cells  
25 remaining in the tissue culture plate, 25  $\mu$ L of a 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/mL) is added to a final concentration of 1 mg/mL. Cells are incubated at 37°C for one hour, and cellular activity is stopped by the addition of an equal volume of MTT lysis buffer (20% w/v sodium dodecylsulfate in 50% dimethylformamide, pH 4.7). Complete extraction is achieved by  
30 overnight shaking at room temperature. The difference in the OD<sub>562nm</sub> and the OD<sub>650nm</sub> is measured in a Molecular Device's UV<sub>max</sub> microplate reader, or equivalent, as an indicator of the cellular viability. Other assays for cell viability include trypan blue exclusion.



### Whole animal-based screening assays

In the whole animal-based screening assays of the present invention, the compound is administered to a transgenic animal of the present invention, over period of time and in various dosages, then the animals are examined for cardiac function, as measured by various parameters including, but not limited to, left ventricular mass:body weight ratio; cardiomyocyte size and organization; cardiac hypertrophy-sensitive gene expression; cardiac function; dP/dT; fibroid deposition; calcium ion flux; stroke length; and ventricular output. A whole-animal based assay can be used as a primary screen, or can be used as a secondary screen to further evaluate substances identified in an enzyme- and/or cell-based screen. In some embodiments, parts of a transgenic animal of the invention can be used in an in vitro screening assay. For example, a heart isolated from a transgenic animal can be used in an in vitro screening assay. Parameters which can be measured are as for whole animal drug screens.

### Assays to detect proteins which interact with enzymes involved in response to a hypertrophic signal

The enzyme targets used in the enzyme-based screening assays described above are also useful for identifying other factors, which may be polypeptides, that interact with the enzyme target. Such factors can then be evaluated for their utility as therapeutic targets in treating a cardiac hypertrophy-induced dysfunction. Such factors can also be analyzed for a potential role in modulating a biological function of the target gene product.

Any known method can be used to identify polypeptides which interact with an enzyme target. Suitable assays include *in vitro* and *in vivo* assays. *In vitro* assays include, but are not limited to, co-precipitation, protein interactive trapping, and ELISA. Assays can be designed to identify compounds that bind to target gene products, bind to other cellular or extracellular proteins that interact with a target gene product, and interfere with the interaction of a target gene product with other cellular proteins.

As an example of protein interactive trapping, a yeast two-hybrid screen can be performed using mouse GATA4 as bait to identify potential interacting factors in the adult myocardium, as described. Molkenstein et al. (1998) *Cell* 93:215-228. In this example, the GATA4 bait contains amino acids 130-409 fused in-frame with the GAL4 DNA binding domain. This region of GATA4 encompasses the two zinc finger domains and is encoded within a PstI - NsiI fragment, which was cloned into a Pst I site in the pAS yeast

expression vector. pAS-GATA4 was co-transformed into yeast with an adult mouse heart library that contained the GAL4 activation domain fused to random cDNAs and over 5 million primary colonies were screened for positive interactions. Approximately 100 positive yeast colonies were initially identified. From each individual colony, the activating plasmid was rescued and the cDNA insert was sequenced. Clones containing cDNA inserts in the antisense orientation or out-of-frame were discarded. The remaining clones (approximately 21) were retransformed back into yeast to test for specificity.

Specificity of binding can be determined using the following criteria: 1) the isolated clone should recapitulate the interaction with the original bait; 2) the isolated clone should not interact with a nonspecific bait, in this case a GAL4-EI2 fusion. As a further test of specificity, one can test interaction with related proteins that share a high degree of amino acid sequence homology.

***Use of transgenic animal or cells derived therefrom***

The transgenic animals produced in accordance with the invention are especially useful for screening and identifying or testing substances or potential drugs for treating hypertrophic cardiomyopathies and/or for research in connection with hypertrophic cardiomyopathies with the object of developing improved therapeutic treatments or diagnostic methods applicable in human medicine.

Substances to be tested include naturally-occurring and synthetic substances. These substances include not only natural and synthetic inorganic and organic compounds based on various core structures, but also oligomers, such as oligopeptides and oligonucleotides. Various natural sources can be screened for active compounds, including those found in jungles, the ocean, and the like. In addition, combinatorial libraries of compounds can be generated and tested. Also to be included for testing are antibodies to a gene product, particularly a polypeptide, of a gene involved in modulating transcription of a cardiac hypertrophy-sensitive gene.

***Compositions comprising substances useful for treatment of cardiac hypertrophy or cardiac failure***

The present invention provides compositions comprising a substance for use in treating a cardiac hypertrophy-induced dysfunction. For the purposes of this invention, a substance for use in treating a cardiac hypertrophy-induced dysfunction is one that

modulates levels of an active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. Substances which are suitable include natural or synthetic organic or inorganic compounds that 1) modulate the expression of a hypertrophy-sensitive gene; 2) modulate the expression of a gene whose product modulates the expression of a hypertrophy-sensitive gene; and/or 3) modulate an activity of a gene product which modulates the expression of a hypertrophy-sensitive gene. A "substance" also includes a polynucleotide that 1) modulates the expression of a hypertrophy-sensitive gene; 2) modulates the expression of a gene whose product modulates the expression of a hypertrophy-sensitive gene; 3) modulates the level of active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

An "effective amount" of a substance is that amount required to achieve amelioration or palliation of cardiac hypertrophy or other condition being treated by the methods of the present invention.

The present invention also encompasses the use of known substances to treat cardiac hypertrophy. Such substances include, but are not limited to, the calcineurin inhibitors FK506 and cyclosporin and related drugs. These drugs are routinely used as immunosuppressants in transplant patients but have not yet been used to treat cardiac hypertrophy or heart failure. "Cyclosporins" are cyclic undecapeptides. A variety of cyclosporins which inhibit phosphatase activity of CaN have been described and are encompassed in the term "cyclosporin". These include, but are not limited to, cyclosporin A; derivatives of cyclosporin A, including, but not limited to, (O-(2-hydroxyethyl)-D-ser)<sub>8</sub> and (3'-deshydroxy-3'-keto-MeBmt)<sub>1</sub>-(Val)<sub>2</sub> derivatives (U.S. Patent Nos. 5,284,826 and 5,525,590); and cyclosporin G. Pharmaceutical formulations of cyclosporin which may be suitable for use in the treatment methods of the present invention include, but are not limited to, those described in UK Patent Publication No. GB 2,257,359; PCT Publication Nos. WO 95/34285 and WO 97/07787; and U.S. Patent Nos. 5,739,105 and 5,641,745. FK506 is a macrocyclic lactone. The term "FK506", as used herein, includes FK506 and derivatives that inhibit a phosphatase activity of CaN, including, but not limited to, derivatives disclosed in U.S. Patent Nos. 5,530,120 and 5,493,019. Also included are substances which are inhibitors of CaMKIV, including KN62, KN93, and related drugs

which inhibit kinase activity of CaMKIV. Further included are inhibitors of p38 kinase, such as those described in PCT Publication No. WO 98/27098.

Substances which have potential for treatment of cardiac hypertrophy and heart failure include substances which enhance the expression of one or more gene products which normally repress transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, thereby reducing the expression of the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal. For example, the transcription factor YY1 has been discovered to be a global repressor of cardiac hypertrophic responsive genes. Over-expression of YY1 in cultured cardiomyocytes prevents cardiac hypertrophy and silences all hypertrophy-sensitive gene expression. Substances which are regulators of YY1 can then potentially be used to regulate cardiac hypertrophy, cardiomyocyte growth and cardiac function. As a further example, constitutively active CaMKII $\alpha$  silences all hypertrophy-sensitive genes in cardiomyocyte cultures. Substances that activate CaMKII $\alpha$  have potential to regulate cardiac hypertrophy.

Substances which have potential for treatment of cardiac hypertrophy and heart failure include also include substances which reduce the expression of one or more gene products which normally increase transcription of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal, thereby reducing the expression of the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

Compositions within the scope of the invention include compositions wherein the active ingredient is contained in an effective amount to alleviate the symptoms of the condition being treated. Determination of the effective amounts can readily be determined empirically by those of ordinary skill of the art.

A substance of the present invention can be administered alone or together with other drug therapies, including,  $\beta$ -adrenergic receptor antagonists, endothelin receptor antagonists, ACE inhibitors, and the like. ACE inhibitors include drugs designated by the trademarks Accupril®, Altace®, Capoten®, Lotensin®, Monopril®, Prinivil®, Vasotec®, and Zestril®.

Pharmaceutical compositions are prepared using a substance or by combining a substance to the appropriate carrier, which itself can be an immunological adjuvant. These

compositions can be administered by any means that achieves the intended purpose. For example, administration can be subcutaneous, cutaneous, intravenous, intradermal, intramuscular, or intraperitoneal.

5 The amount of the substance administered as well as the frequency of administration is dependent upon the age, sex, health and weight of the recipient as well as the nature of the effect desired.

10 In addition to the substance identified by the screening methods of the present invention, these pharmaceutical compositions can contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.1 to 99 percent by weight, and preferably from about 25 to 85 percent by weight, of active ingredient, together with the excipient.

20 The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

25 Suitable excipients are, in particular, fillers such as sugars, for example, lactose, sucrose, mannitol, or sorbitol, cellulose preparations and/or calcium phosphate, such as tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents can be added, such as the above-mentioned starches and derivatives thereof, as well as 30 carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Auxiliaries include flow-regulating agents and lubricants, such as silica, talc, stearic acid or salts thereof such as magnesium or calcium stearate,

and/or polyethylene glycol. Dragee cores can be provided with suitable coatings which, if desired, are resistant to gastric juice. For this purpose, concentrated sugar solutions can be used, which can optionally contain gum Arabic, talc, polyvinyl pyrrolidone, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl-cellulose phthalate or hydroxypropylmethylcellulose phthalate, are used. Dyestuffs or pigments can be added to the tablets or dragee coatings, for example, for identification or in order to characterize different combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which can be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers can be added.

Pharmaceutical preparations which can be administered rectally include suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols, or higher alkanols. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a suitable base. Suitable base materials include, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

A variety of devices are known which allow controlled delivery of a substance to a tissue. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. In addition, suspensions of the active compounds as appropriate oily injection suspensions can be administered. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid

esters, such as ethyl oleate or triglycerides. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension can contain stabilizers.

5       Pharmaceutical excipients are well known in the art and need not be described in detail herein. See, for example, *Remington: The Science and Practice of Pharmacy* (19<sup>th</sup> edition, 1995), Gennaro, ed.

***Methods of treating a cardiac hypertrophy-induced dysfunction***

10       The present invention provides methods for treating a cardiac hypertrophy-induced dysfunction, comprising administering to an individual (a "subject") in need thereof an amount of a substance effective to diminish or reverse progression of the dysfunction. As described above, for use in the methods described herein, a "substance" includes a natural or synthetic organic or inorganic compound that 1) modulates the expression of a hypertrophy-sensitive gene; 2) modulates the expression of a gene whose product modulates the expression of a hypertrophy-sensitive gene; or 3) modulates an activity of a  
15       gene product which modulates the expression of a hypertrophy-sensitive gene.

      As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression,  
20       amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. As used herein, the term "treatment" includes prophylaxis.

      "Palliating" a disease means that the extent and/or undesirable clinical  
25       manifestations of a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not a substance detected by the methods of the present invention.

      The methods comprise administering to an individual (a "subject") in need thereof an amount of a substance effective to diminish or reverse progression of the dysfunction.  
30       In the context of prophylaxis, a "subject in need thereof" includes, but is not limited to, individuals in the general population who are 55 years of age and older; individuals who have a genetic predisposition to developing cardiac hypertrophy; dilated cardiac myopathy

patients; hypertensive patients; patients with renal failure and vascular hypertension; individuals with vascular hypertensive due to pressure overload, volume overload, or increased peripheral bed resistance; individuals with respiratory ailments such as emphysema or cystic fibrosis; chronic asthmatics; individuals with tuberculosis; and organ transplant patients.

In some embodiments, the methods involve blocking an activity of cardiac calcineurin (CaN), particularly with respect to its dephosphorylation action on cardiac transcription factors such as NFAT-3 or other NFAT family members. In some embodiments, the methods involve the use of inhibitors of CaN to block the transcriptional activation of NFAT or other nuclear transcription factors activated by CaN-mediated dephosphorylation. In other embodiments, the methods involve inhibition of CaN by clinically approved specific inhibitor drugs, including members of the immunosuppressive family of cyclosporin A derivatives, to reduce the dephosphorylation activity of CaN, in the cytosol and/or the nucleus of the cardiomyocyte. Blocking the dephosphorylation of NFAT transcription factors by immunosuppressive drugs which specifically inhibit CaN substantially reduces or prevents translocation of these transcription factors to the cardiomyocyte nuclear compartment, rendering them incompetent to form an active heteroduplex with GATA4. The inability to form the NFAT-3/GATA-4 heteroduplex or similar complexes between activated NFAT members and GATA transcription factors prevents such complexes from binding to their specific enhancer sites on a number of hypertrophy-sensitive cardiac promoters. Since a number of cardiac hypertrophy-sensitive promoters contain such binding sites and are responsive to NFAT-3/GATA-4 or similar complexes, their gene products can be up-regulated by CaN activity and therefore can be inhibited by CaN-specific immunosuppressive inhibitors.

CaN-specific immunosuppressive inhibitors block most nuclear signaling converging through CaN due to cardiovascular pressure overload-induced cardiac hypertrophy and block the transcriptional induction leading to chronic hypertrophy, in turn leading to dilated cardiac myopathy in the chronic untreated animal. In other embodiments, the use of inhibitors of CaN blocks all pressure overload membrane receptor-mediated nuclear signaling for the transcriptional induction of cardiac hypertrophy. Receptor-mediated transcriptional induction of cardiac hypertrophy through pressor agents, Angiotensin II (AngII), Endothelin-1 (ET-1) thrombin (Thrb) and alpha



adrenergic signaling through the alpha-1-adrenergic receptor converge as a nuclear transcription activation signal at CaN. Because these pathways converge at CaN, pressor agent- and alpha-1-adrenergic receptor-mediated transcriptional induction of hypertrophy-sensitive cardiac genes can be blocked by CaN inhibitors.

5 In other embodiments, the methods inhibit the expression of gene products which are expressed in response to CaN dephosphorylation of NFAT transcription factors, i.e., gene products of hypertrophy-sensitive genes. In some of these embodiments, the agent that inhibits expression of one or more hypertrophy-sensitive genes is an antisense polynucleotide which targets a hypertrophy-sensitive gene.

10 In embodiments which comprise administration of an agent(s) which inhibits CaN, thereby blocking the dephosphorylation of NFAT-3 and/or one or more related family members, an agent that blocks CaN is a member of the cyclosporin family of immunosuppressive drugs, such as cyclosporin A (CsA) or FK506. Any agent that inhibits cardiac CaN activity to block the dephosphorylation of one or more NFAT  
15 transcription factor family members may prove useful.

In some embodiments, the methods administer an agent that reduces expression of cardiac CaN. Such agents include, but are not limited to, a dominant negative form of CaN (e.g., negative truncated form of the coding region of the alpha subunit of CaN); an antisense construct which targets a CaN gene; and a truncated, inactive form of the beta  
20 subunit of CaN. A truncated, inactive form of the  $\beta$ -subunit of CaN can sequester the active alpha subunit of CaN, and render it incompetent for activation. In other embodiments, the methods comprise administering an antibody specific for CaN, or the alpha or beta subunit thereof. In some of these embodiments, the antibody is a single chain antibody. In other embodiments, the antibody is an antibody that blocks CaN  
25 activity or prevents NFAT-3 and/or an NFAT family member from being dephosphorylated by cardiac CaN.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose  
30 therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds which exhibit large therapeutic indices are preferred. While compounds that

exhibit toxic side effects may be used, a delivery system can be designed that targets such compounds to the site of affected tissue in order to minimize potential damage to non-target cells and, thereby, reduce side effects.

5 The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell  
10 culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC<sub>50</sub> (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture and/or in whole animals. Such information can be used to more accurately determine useful doses in humans.

15 The therapeutic efficacy of a substance detected by the methods of the present invention in the treatment of cardiac hypertrophy can be accomplished by those skilled in the art using known principles of diagnosis and treatment.

The following examples are provided to illustrate, but not to limit, the present  
20 invention.

#### EXAMPLE 1

##### *Inhibition of the hypertrophic effects of AngII and PE by CsA and FK506 in primary cardiomyocytes*

##### Experimental protocol

  
25

To visualize primary cardiomyocytes and their sarcomeric organization, anti- $\alpha$ -actinin mouse monoclonal antibody was used (Sigma). This antibody is specific for the cardiac and skeletal  $\alpha$ -actin proteins. Cells were washed in 1X phosphate-buffered saline (PBS), fixed in 3.7% paraformaldehyde for 5 minutes, washed three times with 1X PBS  
30 and then pre-blocked in 1X PBS containing 2% horse serum, 2% BSA, and 0.1% non-ionic detergent NP40 for 30 minutes. Anti- $\alpha$ -actinin antibody was added at a dilution of 1:800 in fresh preblock solution and incubated for an additional 30 minutes. Subsequently, the

cells were washed three times in 1X PBS with 0.1% NP40. Anti-mouse TRITC-conjugated secondary antibody was then added at a dilution of 1:400 for 30 minutes in pre-block solution and the cells were again washed three times in 1X PBS containing 0.1% NP40. Nuclear staining for DNA was performed with 0.5 µg/ml of bisbenzimidazole in PBS for 15 minutes followed by three rinses with PBS.

### Results

Exposure of primary cardiomyocytes to AngII and PE results in a pronounced hypertrophic response, which is characterized by an increase in cell size and sarcomeric assembly, upregulation of several fetal contractile protein genes, and enhanced contractility. These events are preceded by an increase in intracellular calcium. To determine whether the hypertrophic response of cardiomyocytes to these agonists was mediated by calcineurin, neonatal rat cardiomyocytes were exposed to AngII (10nM) or PE (10 µM) in the presence and absence of CsA or FK506. Cardiomyocytes demonstrated a dramatic increase in size and sarcomeric assembly after 72 hr of exposure to AngII or PE (Figures 2B and 2E). In the presence of CsA (Figures 2C and 2F) or FK506, the response to AngII was completely abolished and the response to PE was dramatically reduced.

To determine whether changes in cardiomyocyte gene expression in response to AngII were also controlled by a calcineurin-dependent signaling pathway, the expression of ANF mRNA in cardiomyocytes treated with AngII was examined in the presence and absence of CsA by a dot blot assay. Exposure to AngII resulted in a 15-fold increase in atrial natriuretic factor (ANF) mRNA, which was completely blocked by CsA (Fig. 23G). GAPDH mRNA was measured as a control. Together, these morphologic and molecular data demonstrate that the AngII and PE signaling pathways are CsA-/FK506-sensitive. Because calcineurin is the only known target for inhibition by both CsA and FK506, these results suggest that calcineurin activation is a necessary step in the signaling pathway for AngII- and PE-dependent induction of cardiac hypertrophy.

## EXAMPLE 2

### *Induction of cardiac hypertrophy in vivo*

#### Experimental protocols

**Transgenic mice.** Transgenic mice expressing calcineurin in the heart were created as follows. A cDNA encoding a constitutively active form of the calcineurin A catalytic subunit (O'Keefe et al. (1992) *Nature* 357:692-694) was cloned by PCR with a 5'

Sall linker and 3' HindIII linker into an expression vector containing the  $\alpha$ -MHC promoter. The expression pattern and characteristics of this expression vector have been described previously (Jones et al. (1994) *Dev. Dyn.* 22:117-128). The calcineurin- $\alpha$ -MHC vector was digested with NotI to liberate the pBluescript backbone and allow purification of the  
5  $\alpha$ -MHC-fusion cDNA fragment. This fragment was purified on an agarose gel using the Qiaex II gel extraction kit (Qiagen) and eluted in oocyte injection buffer (5 mM Tris - HCl pH 7.4 and 0.2 mM EDTA). The purified fragment was injected into recently fertilized oocytes derived from FVB mice and transferred into the oviducts of pseudopregnant ICR mice.

10 Transgenic mice expressing CaMKIV in the heart were created as follows. A cDNA encoding a constitutively active form of CaMKIV (O'Keefe et al. (1992) *Nature* 357:692-694) was cloned by PCR and inserted into an expression vector containing the  $\alpha$ -MHC promoter, as described above for the calcineurin mouse. DNA was prepared for injection into oocytes, and transgenic mice were generated, as described above.

15 **RNA analysis.** Total RNA was collected and purified with Triazol reagent (Gibco BRL) as recommended. RNA from wild-type and transgenic hearts, as well as from cultured cardiomyocytes, was subjected to dot blot hybridization against a panel of oligonucleotide probes as described previously (Jones et al. (1996) *J. Clin. Invest.* 98, 1906-1917).

20 **Histology.** Hearts from wild-type and transgenic mice were subjected to histological analysis. Briefly, hearts were collected, fixed overnight in 10% formalin buffered with PBS, dehydrated in ethanol, transferred to xylene then into paraffin. Paraffin-embedded hearts were sectioned at 4  $\mu$ m and subsequently stained with hematoxylin and eosin for routine histologic examination or with Masson trichrome for collagen (Woods and Ellis In, *Laboratory Histopathology: A Complete Reference* (1994)  
25 Churchill Livingstone Publishers pp. 7.1-13).

### Results

**Induction of cardiac hypertrophy in vivo by activated calcineurin.** The above results indicated that calcineurin was a potent regulator of cardiac hypertrophy in cultured  
30 primary cardiomyocytes. To determine whether this signal transduction pathway could also operate in the myocardium *in vivo*, transgenic mice were generated that expressed the constitutively active form of the calcineurin catalytic subunit in the heart, using the  $\alpha$ -

MHC promoter to drive expression. Previous studies have shown that this cardiac-specific promoter is active in the ventricular chambers primarily after birth (Jones et al. (1994) *Dev. Dyn.* 22:117-128). A total of 10 independent founder transgenic mice were generated, which contained between 2 and 68 copies of the  $\alpha$ -MHC-calcineurin transgenic (Table 1).

**Table 1: Summary of  $\alpha$ -MHC-Calcineurin Transgenic Lines**

| <u>Transgenic Line</u> | <u>Transgene Copy</u> | <u>Cause of Death</u> | <u>Age at Death</u> | <u>Heart: Body wt.</u> | <u>Cardiac Phenotype</u> |
|------------------------|-----------------------|-----------------------|---------------------|------------------------|--------------------------|
| 46                     | 8                     | Sacrificed            | 18 days             | 2.2                    | Hypertrophic             |
| 22                     | 22                    | Sudden                | 10 weeks            | 2.3                    | Dilated                  |
| 110                    | 3                     | Sudden                | 4 weeks             | 2.6                    | Hypertrophic             |
| 106                    | 2                     | Sudden                | 9 weeks             | N.D.                   | N.D.                     |
| 108                    | 3                     | Still alive           | (14 weeks)          | -                      | -                        |
| 41                     | 68                    | Still alive           | (24 weeks)          | -                      | -                        |
| 37                     | 15                    | Still alive           | (23 weeks)          | -                      | -                        |
| 37-1                   | 15                    | Sacrificed            | 5 weeks             | 2.3                    | Hypertrophic             |
| 37-2                   | 15                    | Sudden                | 4 weeks             | N.D.                   | Hypertrophic             |
| 37-3                   | 15                    | Sudden                | 3 weeks             | 2.5                    | Hypertrophic             |
| 37-4                   | 15                    | Still alive           | (8 weeks)           | -                      | -                        |
| 37-5                   | 15                    | Still alive           | (8 weeks)           | -                      | -                        |
| 37-6                   | 15                    | Sudden                | 12 weeks            | 2.9                    | Dilated                  |
| 39                     | 3                     | Sudden                | 11 weeks            | 2.7                    | Hypertrophic             |
| 39-1                   | 3                     | Sudden                | 3 weeks             | N.D.                   | Hypertrophic             |
| 39-2                   | 3                     | Sudden                | 4 weeks             | N.D.                   | N.D.                     |
| 39-3                   | 3                     | Still alive           | (10 weeks)          | -                      | -                        |

Heart:body weight (heart:body wt) ratios were calculated by weighing the hearts and bodies of nontransgenic and transgenic litter mates. Values are expressed as the relative weight of the transgenic heart compared to nontransgenic litter mate. Ages of mice that were still alive as of February 18, 1998 are shown in parenthesis. 37 and 39 were founder transgenics and mice designated as 37- and 39- were their offspring. The term "N.D." indicates that this value was not determined.

Every calcineurin transgenic mouse analyzed showed a dramatic increase in heart size relative to nontransgenic littermates. The heart-to-body weight ratio averaged 2- to 3-fold greater in the calcineurin transgenics compared to control littermates, even as early as

18 days postnatally (Figure 3; Table 1). No difference in the cardiac phenotype in males and females was observed. Histological analysis showed concentric hypertrophy wherein the cross-sectional areas of the ventricular walls and interventricular septum were dramatically increased (Figure 3C). The left ventricle was most affected, but the right ventricle and the atrial chambers were also enlarged. In contrast to the well-organized, striated musculature of the normal ventricular wall, cardiomyocytes from the calcineurin transgenic hearts were disorganized and obviously hypertrophic (Figures 3D and 3E). The hypertrophic cardiomyocytes often had dramatic karyomegaly. Measurement of cross-sectional areas of myocytes within the left ventricular wall showed a greater than 2-fold increase in calcineurin transgenics compared to controls.

In humans, cardiac hypertrophy frequently progresses to ventricular dilatation, heart failure and sudden death. Similarly, in calcineurin transgenic mice, dilatation of the ventricular chambers with increasing age was observed (Figures 3F and 3G). Calcineurin transgenic mice were also highly susceptible to sudden death. This occurred spontaneously, as well as during handling or anesthesia. The mice that died from sudden death showed right and left ventricular dilatation indicative of heart failure. Histology of the lungs also revealed extensive perivascular edema and intra-alveolar macrophages containing red blood cells, findings consistent with heart failure. One of the hallmarks of heart failure is fibrosis of the ventricular wall. The hearts of calcineurin transgenics contained extensive, primarily interstitial, deposits of collagen, as revealed by trichrome staining (Figure 3H). In foci with marked fibrosis, myofiber degeneration was evident.

Similarly, transgenic mice expressing a constitutively active form of CaMKIV under control of an  $\alpha$ -MHC promoter, showed dramatic increases in heart size relative to nontransgenic littermates, as shown in Figure 5.

**Activation of the molecular response to hypertrophy in vivo by calcineurin.** To determine whether activated calcineurin induced changes in cardiac gene expression characteristic of hypertrophy and heart failure, a quantitative dot blot assay was used to examine RNA from hearts of calcineurin transgenics and nontransgenic littermates. Consistent with the reactivation of the fetal program of gene expression, MHC,  $\alpha$ -skeletal actin, and *BNP* transcripts were dramatically upregulated in transgenic hearts, whereas  $\alpha$ -MHC was downregulated. Transcripts for sarcoplasmic reticulum  $\text{Ca}^{++}$ -ATPase (SERCA) and phospholamban (PLB) have been shown previously to be downregulated during heart

failure, as the failing myocardium exhibits defective  $\text{Ca}^{++}$ -handling (Mercadier et al. (1995) *J. Clin. Invest.* 85:305-309; Schwinger et al. (1995) *Circulation* 92:3220-3228); both transcripts were decreased in calcineurin transgenics. There was no significant change in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

5           **Prevention of cardiac hypertrophy with CsA.** To determine whether inhibition of calcineurin activity *in vivo* might be an effective means of preventing cardiac hypertrophy, we tested whether subcutaneous injection of CsA could prevent cardiac dysfunction in calcineurin transgenic mice. For these experiments, 8 transgenic littermates from a litter of transgenic mouse #37 (Table 1) were used.

10           Four transgene-positive offspring were injected twice daily with 25 mg/ml CsA and four were injected with vehicle alone. Four nontransgenic littermates were also treated with CsA to control for potential toxic effects or any cardiac abnormalities induced by CsA. CsA treatment was initiated at 9 days of age and animals were sacrificed 16 days later. As shown in Figure 4C, the hearts of vehicle-treated animals were highly  
15           hypertrophic and dilated by day 25, whereas those from CsA-treated littermates were not significantly different in size from nontransgenic controls. The mean heart-to-body weight ratios for calcineurin transgenics were nearly 3-fold larger than those of CsA-treated transgenics and nontransgenics, as shown in Figure 4B. CsA treatment also prevented fibrosis of the hearts of calcineurin transgenics.

20           At a cellular level, the hypertrophic response of cardiomyocytes in the calcineurin transgenics was largely inhibited by CsA, although there were isolated areas of myofiber disarray and scattered cells with prominent hyperchromatic nuclei. CsA treatment prevented gross cardiac hypertrophy and associated pathology in response to activated calcineurin *in vivo*.

### 25           EXAMPLE 3

*Characterization of effects of CaMKII and CaN on expression of hypertrophy-sensitive genes in a cultured cardiomyocyte cell model*

#### **Protocols**

30           **Primary myocardial cell cultures.** Primary cultures of neonatal rat cardiac myocytes were prepared using 1-4 days old Sprague-Dawley rats. Rat pups (an average of 15 pups per preparation) were decapitated and hearts were removed sterilely from thoracic cavity and washed with Medium 199 (Life Technologies, Gaithersburg, MD) to remove

residual blood. The atria were removed and ventricles were finely minced and subjected to three sequential Viokase (A. H. Robbins Company, Richmond, VA) digestion of 18 minutes each. Viokase containing lipase, protease and amylase as well as other pancreatic enzymes was made at 1 mg/ml in PBS buffer and used at a rate of 120 ml per 15 hearts.

5 Supernatants pooled from the three digestions were put on ice bath in order to quench the digestion reaction. The supernatants were then centrifuged at 2000 rpm for 8 min at room temperature in a Beckman TJ-6 rotor. Cell pellets were resuspended in Medium 199 supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. Cells were then plated in 100-mm tissue culture dishes and incubated in a 37°C cell culture incubator.  
10 This process, called "pre-plating", selectively removes rapidly attaching, non-cardiac myocyte cells such as fibroblasts from the cell population therefore enriching the cell population for cardiomyocytes. Forty-five to fifty minutes later, the non-attached cells were transferred to fresh 60-mm dishes at a density of about 150 cells/mm<sup>2</sup> for the initiation of long-term culture. After 20-24 hours, myocardial cell cultures were washed  
15 and maintained in fresh medium supplemented with 10% fetal bovine serum as described above in 60-mm dishes. The cultures of cardiomyocyte could be maintained for up to 7 days.

**Transient transfection.** It was necessary to culture the isolated cardiomyocytes in serum-free medium to eliminate effects on gene expression mediated through serum  
20 response elements. Primary myocardial cultures were first washed with serum-free growth medium and then maintained in serum-free Medium 199 supplemented with Nutridoma HU (Boehringer Mannheim Corporation, Indianapolis, IN) which contained human serum albumin, bovine insulin and human transferrin. Transfection were carried out by using a liposome-based transfecting agent, Lipofectamine (Life Technologies, Grand Island, NY).  
25 The procedures used for transient transfection were followed as the manufacturer's instructions. The optimal values including DNA concentration, lipofectamine concentration, and time length for transfection were determined prior to experiments carried out in this study. Briefly, for each transfection, 2 µg DNA and 20 µg lipofectamine were diluted into separate vials containing 300 µl serum-free nutridoma supplemented  
30 medium. The two solutions were then combined and incubated at room temperature for 15-45 minutes to allow DNA-liposome complexes to form. For each transfection, 2.4 ml of serum-free nutridoma supplemented medium were added to the tube containing the



DNA-liposome complexes and the diluted mixture was overlaid onto the adherent cardiomyocytes that had been rinsed once with 2 ml of serum-free medium in a 60-mm tissue culture plate. The cell cultures were incubated at 37°C in a CO<sub>2</sub> incubator containing 5% CO<sub>2</sub> for 24 hours, at which time various test compounds were added.

5 Promoter activities from cell extracts were assayed within 48 hours following the start of transfection.

For  $\alpha_1$ -adrenergic stimulation, 100  $\mu$ M PE (Sigma, St. Louis, MO) was added to the cells. DOB (Gensia Laboratories, Ltd., Irving, CA), a selective  $\beta_1$ -adrenergic agonist, was added at a concentration of 10  $\mu$ M. To correct for variation in transfection  
10 efficiencies, p $\beta$ Gal-Control was used as an internal control. Chemiluminescent  $\beta$ -galactosidase activity was assayed by using an aliquot of the same cell lysates collected for the luciferase assay and measured in the same luminometer as described below.

**Firefly luciferase assay.** The firefly luciferase assay is based upon a commercial (Promega, Madison, WI) long term "glow" type of bioluminescence assay. Briefly,  
15 activation of luciferin results in the formation of an enzyme-bound luciferyl adenylate (luciferyl-AMP). The enzyme intermediate, luciferyl-AMP is oxidated in the presence of coenzyme A (CoA) and leads to the formation of an enzyme-bound excited product which subsequently decomposes to emit light and a tightly enzyme-bound product. In the presence of CoA, oxidation occurs instead from luciferyl-CoA with more favorable total  
20 kinetics. Cells to be assayed for luciferase activity were rinsed twice with PBS buffer and lysed in Reporter Lysis 1X Buffer (Promega) and then harvested by scraping. Large debris were pelleted by brief centrifugation and supernatant was transferred to fresh tubes. For luciferase assay, 20  $\mu$ l of cell extract was mixed with 100  $\mu$ l Luciferase Assay Reagent (Promega) which contained 20 mM Tricine, 1.07 mM (MgCO<sub>3</sub>)<sub>4</sub>Mg(OH)<sub>2</sub> 5H<sub>2</sub>O, 2.67 mM  
25 MgSO<sub>4</sub>, 0.1 mM EDTA, 33.3 mM DTT, 270  $\mu$ M coenzyme A, 470  $\mu$ M luciferin, and 530  $\mu$ M ATP. Integrated light emission over 10 seconds to 5 minutes was determined in a Turner Designs Luminometer Model 20 (Promega). All assays were performed at room temperature within 1 hour of cell lysis.

**Northern Analysis.** For ANF message analysis, total RNA were extracted from 1-  
30 3-day-old neonate rat cardiomyocyte cell cultures according to the method of Chomczynski and Sacchi using the commercially available TRIzol Reagent (Life

Technologies, Grand Island, NY). Rat primary cell cultures were homogenized in TRIzol, and the RNA was extracted by the addition of chloroform and precipitated in the presence of isopropanol. The isolated total RNA were fractionated on a 1% agarose/formaldehyde gel, and transferred to a nylon membrane by capillary blotting. Blots were prehybridized in a 50% formamide solution (Life Technologies, Grand Island, NY) for 3-4 hours at 42°C and then hybridized overnight with a random primer, biotin-labeled ANF cDNA probe. After washing at high stringency (0.1 X SSPE, 0.1% SDS, 65°C), hybridized biotinylated cDNA were detected by using Chemiluminescent Detection System (TROPIX, Inc., Bedford, MA) and then exposed to X-ray film. The ANF bands were quantitated with a Howteck gel scanner (PDI, Huntington station, NY) and normalized to the 28 S rRNA signal to correct for loading and/or transfer efficiencies.

**Determination of autonomous CaM kinase II activity.** CaM kinase II autonomous activity was determined in rat heart tissue homogenates using the synthetic peptide substrate, autocamtide-2 (KKALRRQETVDAL) (Mar et al. (1990) *Mol. Cell. Biol.* 10:4271-4283; and Farrance et al. (1992) *J. Biol. Chem.* 267:17234-17240) as exogenous substrate. This peptide was modeled after the core autophosphorylation sequence of the rat brain  $\alpha$ -CaM kinase II and is highly selective for CaM kinase II. Autonomous CaM kinase II activity was assayed by the phosphorylation of autocamtide-2 in the absence of  $\text{Ca}^{2+}$ /calmodulin and expressed as a proportion of activity obtained in the presence of these cofactors. Tissue slices were incubated in the presence or absence of various drug compounds for required time. At termination of the incubation the tissue slices were homogenized in ice-cold buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1.0 mM EDTA, 2.0 mM dithiothreitol, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate, 100 mg/ml leupeptin) by sonication. Supernatant were cleared by centrifugation (12,000 x g, 2 minutes) and then assayed in a reaction mixture containing 50 mM PIPES (pH 7.0), 10 mM  $\text{MgCl}_2$ , 0.1 mg/ml bovine serum albumin, 10 mM autocamtide-2, 20 mM  $[\gamma\text{-}^{32}\text{P}]$  ATP and either 0.5 mM  $\text{CaCl}_2$ , 5 mg/ml calmodulin for  $\text{Ca}^{2+}$ -stimulated activity or 1 mM EGTA for autonomous activity. The assay was conducted at 30° C for 30 seconds and terminated by the addition of trichloroacetic acid to a final concentration of 5%. Endogenous protein was sedimented by centrifugation and supernatants containing autocamtide-2 were spotted onto p81 phosphocellulose strips, washed 5 times, dried and  $^{32}\text{P}\text{-PO}_4$  content was quantitated by Cerenkov radiation.

**Plasmid constructs.** A series of nested deletion mutants containing various rat cardiac ANF promoter fragments and the rat ANF cDNA were used. This series of promoter-reporter constructs were subcloned into luciferase-based ppx2 vectors. The 5' deletions were generated using appropriate restriction enzyme sites or by PCR amplification. A diagram of key enhancer elements thought to regulate ANF transcription is shown in Figure 8A. The skeletal  $\alpha$ -actin reporter, harboring approximately 400 bp of proximal upstream promoter domain (-394 bp to +24 bp relative to the transcription start site) of the avian cardiac skeletal  $\alpha$ -actin gene (containing the majority of the enhancer domains responsive to growth signaling linked to the structural portion of the firefly luciferase gene) was used. Another luciferase-based promoter-reporter construct harboring 400 bp of proximal upstream promoter domain of the avian cardiac  $\alpha$ -actin gene was also used. Two isoforms of  $\delta$ -CaM kinase ( $\delta_A$  &  $\delta_B$ ) harbored in pSR $\alpha$  expression vector were used. Expression plasmids for CaM kinase IV including both a full-length form (CaMKIVwt) and a mutated form (CaMKIV313) were used. The constitutively-active form of calcineurin, from neuronal origin, was generated by polymerase chain reaction mutagenesis technique which resulted in the deletion of the auto-inhibitory domain of calcineurin. The mutated fragment was then subcloned into pSR $\alpha$  expression vector. The expression plasmid for mutant brain  $\alpha$ -CaM kinase II was constructed by subcloning a cDNA encoding a truncated version (amino acids 1-290) of the wild-type CaM kinase II enzyme which is constitutively active into the EcoRI site of pCMV5, a mammalian expression plasmid containing a multiple cloning sequence immediately down stream from the strong viral promoter of cytomegalovirus. This construct, when transfected into the cardiomyocyte cultures, constitutively produces CaM kinase II enzyme which does not bind  $Ca^{2+}$  or calmodulin.

## Results

### Effects of exogenous CaM kinase II and calcineurin on promoter-reporter activities for ANF, skeletal $\alpha$ -actin and cardiac $\alpha$ -actin genes

In this study, we explored a potential functional role for a calcium-dependent protein kinase, CaM kinase II (CaMKII), and a calcium-sensitive phosphatase, calcineurin (CaN), in regulating ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin gene expression. Three luciferase-based promoter-reporter constructs containing nearly full length ANF, skeletal

$\alpha$ -actin or cardiac  $\alpha$ -actin promoter regions as described above were employed in this study. A co-transfection protocol using vector constructs harboring a mutant, constitutively-active CaMKII  $\alpha$ -isoform or a constitutively active mutant form of CaN with the ANF/luciferase, skeletal  $\alpha$ -actin/luciferase or cardiac  $\alpha$ -actin/luciferase fusion genes was carried out on neonate cardiomyocyte cultures prepared from 1-4-day-old rat pups. Luciferase reporter activities were evaluated. As shown in Figure 6, over-expression of the exogenous CaMKII  $\alpha$ -isoform silenced promoter-reporter activities for ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin by 50% compared to control cultures (co-transfected with empty vector). In contrast, over-expression of the exogenous constitutively-active CaN increased ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin promoter activities by 300%-600% over control cells. Results in this experiment suggested that overexpression of a heterologous CaMKII  $\alpha$ -isoform leads to silencing of promoter activities for both the cardiac embryonic genes, ANF & skeletal  $\alpha$ -actin, and constitutive contractile protein gene, cardiac  $\alpha$ -actin. In contrast, activation of a exogenous CaN results in superinduction of the promoter activities for these three genes.

#### Effects of CaM kinase isoenzymes on ANF promoter-reporter activity

The CaM kinase family has at least ten members, which differ in structure, distribution, regulation and enzymatic activity. The results, presented in Figure 6, suggest that mutated  $\alpha$ -CaMKII induced down-regulation of hypertrophy-sensitive genes. It was of interest to determine what effects of other CaM kinase family members play on the prototypical hypertrophy-sensitive gene, ANF. Select members of CaM kinase family, including wild type CaMKII (CaMKII<sub>wt</sub>),  $\delta$ -CaMKII ( $\delta_A$  &  $\delta_B$ ), and CaMKIV (wild-type & mutated form), were used for co-transfection with the ANF promoter-reporter construct described above. PE induction of ANF was also evaluated in the presence of each of the exogenous CaM kinase members. As shown in Figure 7, CaMKII<sub>wt</sub> reduced ANF promoter activity by ~400%. In contrast, CaMKIV<sub>wt</sub> increased ANF promoter activity by 270%, while CaMKIV<sub>mut</sub> increased ANF promoter activity by ~400%. CaMKII  $\delta$  isoforms including both  $\delta_A$  and  $\delta_B$  virtually did not alter the promoter activities of ANF. Similar results were obtained in the presence of PE stimulation in transfected cardiomyocytes, as shown in Figure 7B. Taken together, these data indicate that different CaM kinase family members show differential effects on the expression of ANF.

Effect of exogenous CaMKII or CaN on ANF promoter-reporter nested deletion constructs

In these studies, a series of nested deletion constructs generated from the 5' promoter/enhancer domain of the rat cardiac ANF gene was used. Three different lengths of the native ANF promoter fragments were ligated to the structural portion of the firefly luciferase gene as described above. A diagram of all of the identified functional enhancer elements for ANF gene is shown in Figure 8A. Reporter activity for each of the nested deletion constructs in cardiomyocyte cultures exposed to PE is shown in Figure 8B. Promoter-reporter constructs containing sufficient upstream sequence to include the PERE (PE Response Element) motif, ANF700 (NP337) and ANF135 (NP325), but not promoter sequences which have lost this motif, ANF50 (NP338), were activated by PE exposure. It was assessed whether silencing/induction of the ANF promoter-reporter activity by expression of exogenous CaMKII/CaN could be localized to a region of the ANF promoter domain. The ANF promoter-reporter nested deletion constructs described above were co-transfected respectively with constitutively-active CaMKII or CaN. As shown in Figure 8C, over-expression of exogenous CaMKII resulted in silencing of all three ANF 5'-deletion promoter constructs by 50% compared to control cultures, whereas over-expression of exogenous CaN increased each of the three ANF promoter construct by a range from 200-600% over control cultures. These data indicate that inhibition/induction of the ANF promoter-reporter activity by CaMKII/CaN could not be restricted to a defined region or domain for the ANF promoter.

Beta-adrenergic receptor signaling and cardiac CaM kinase II activation

These experiments determined whether signaling through  $\beta_1$ -adrenergic receptor subtype could activate cardiac CaMKII. Heart tissue slices from 1-4-day-old neonatal rats were freshly isolated and treated with either the  $\beta_1$  selective agonist, dolbutamine (DOB), or the mixed  $\beta_1$  ( $\beta_1$  &  $\beta_2$ ) agonist, isoproterenol (ISO). In parallel studies,  $\beta_1$  antagonist, propranolol (PROP) was added 15 sec before agonists were added. Later, autonomous activity (% of calcium independent activity) of CaMKII was assayed. The results of such an experiment are shown in Figure 9. CaMKII autonomous activation was increased ~170% over the activity observed in unstimulated heart tissues 30 seconds following treatment by DOB or ISO. In addition, the  $\beta_1$  antagonist, PROP, when added prior to either DOB or ISO addition, blocked activation of CaMKII autonomous activity. These

results demonstrate that exposure to  $\beta_1$ -adrenergic agonist leads to cardiac endogenous CaMKII activation. This activation of CaMKII activity is a  $\beta_1$ -receptor-mediated response.

Effect of adrenoceptor agonists on promoter-reporter activities for ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin genes

Results of experiments shown above suggested that CaN leads to induction of these three genes in neonate rat cardiomyocytes. It was of interest to determine the effect of  $\alpha$ - and  $\beta$ -adrenergic stimuli on the expression of ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin. Luciferase-based promoter-reporter constructs harboring ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin promoter domains as described above were transfected into primary neonatal rat cardiomyocyte cultures. The transfected cardiomyocytes were then exposed to the  $\alpha_1$ -adrenergic agonist, phenylephrine (PE),  $\beta_1$ -adrenergic agonist, DOB, or type  $\beta$ -transforming growth factor (TGF- $\beta$ 1). As shown in Figure 10, panels A, B and C, each of these promoter-reporter activities was decreased approximately 50% by treatment with DOB. Reporter activities for ANF and skeletal  $\alpha$ -actin were transactivated by 200% over that in control cells when cardiomyocytes were exposed to PE, as shown in Figure 10, panels A and B. Promoter activity for cardiac  $\alpha$ -actin was not significantly stimulated in PE-treated cardiomyocytes, whereas treatment with TGF- $\beta$ 1, in agreement with previous studies, induced cardiac  $\alpha$ -actin promoter-reporter activity by 700% over control cells. These data show that both  $\alpha$ -adrenergic and  $\beta$ -adrenergic stimulation modulates the expression of cardiac hypertrophy-sensitive genes, such as ANF, skeletal  $\alpha$ -actin and cardiac  $\alpha$ -actin.  $\beta_1$ -adrenergic stimulation is able to inhibit the promoter activities for both embryonic genes, like ANF, skeletal  $\alpha$ -actin, and constitutive contractile protein gene, like cardiac  $\alpha$ -actin. Therefore, it was of interest to assess whether the changing of ANF reporter activity observed in the above experiments was a response to the alteration of ANF message pools.

Primary cultures of neonatal cardiocytes were kept in serum-free medium and  $\beta_1$  agonist, DOB, was applied to the cells at various times. ANF message RNA was evaluated in the end by northern analysis protocols as described above. As shown in Figure 10D, when cell cultures were exposed to DOB, abundance of the ANF message began to drop so that within 16 hours it was only about half that observed in control

cultures (no drug addition). This trend continued so that 24 hours after DOB exposure, ANF mRNA had declined by 75% compared to control values. These results suggest that part, if not all, of the changes in reporter activities occurred at the level of gene transcription.

#### EXAMPLE 4

##### *Transcriptional regulation of hypertrophy-sensitive genes by an inducible calcineurin expression system*

###### Plasmids Constructs

Tetracycline-controlled transactivator (tTA) expression plasmid pUHD15-1 encodes chimeric protein tTA under the control of the human cytomegalovirus (CMV) promoter and enhancer. tTA consists of amino acid residues 1-207 from the tetR protein and residues 363-490 from VP16 (Gossen and Bujard (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551).

Plasmid pUHD10-3 contains a chimeric tet operon/CMV minimal promoter upstream of multiple cloning sites (Bujard, 1996). pUHC13-3 is a firefly luciferase reporter gene expression plasmid driven by the chimeric tet operon/CMV minimal promoter (Gossen and Bujard (1992)).

A constitutively active gene form of calcineurin ( $\Delta$ CaM-AI) was originally recovered from plasmid pCN( $\alpha$ ) $\Delta$ CaM-AI, which was generated by subcloning the auto-inhibitory domain-deleted calcineurin gene mutant into pSR $\alpha$  expression vector (Parson et al., 1994). To generate tTA inducible calcineurin construct p10-3CaN, a 1.2 Kbp EcoRI fragment of pCN( $\alpha$ ) $\Delta$ CaM-AI, coding the constitutively active calcineurin, was subcloned into the unique EcoRI cleavage site of the plasmid pUHD10-3, which contains a tTA-dependent cytomegalovirus (CMV) minimal promoter in front of the EcoRI cloning site (Bujard, 1996).

ANF-luciferase promoter-reporter construct, NP337 was made by cloning about 700-bp nucleotides of ANF proximal promoter fragment into luciferase-based pXP-2 vector as previously described elsewhere (McBride et al., 1993).

The skeletal  $\alpha$ -actin reporter, SkA-Luc, was constructed by subcloning approximately 420bp proximal upstream promoter (-394 bp to +24 bp) of avian skeletal  $\alpha$ -actin gene into the firefly luciferase expression vector pXP1 (MacLellan et al., 1994).

Another reporter construct, cardiac  $\alpha$ -actin promoter-luciferase (CardA-Luc), was generated by subcloning a 330-bp fragment of chicken cardiac  $\alpha$ -actin promoter and immediate upstream region (from -315 to +15, relative to the transcription start site) into the HindIII cloning site of the pGL2-basic luciferase vector (Chen and Schwartz, 1996).

## 5 Cell Cultures

Primary myocardial cell cultures Primary ventricular myocytes were prepared from 1-4 day old Sprague-Dawley rats, as described in Example 3.

## Results

### 10 *Regulation of an exogenous target gene transcription by the tetracycline-dependent transactivator (tTA) expression system in primary neonatal rat cardiomyocytes.*

The firefly luciferase gene as a reporter gene was under control of the chimeric tet operon/CMV minimal promoter in pUHC13-3. Transient transfections were carried out as described in Example 3. When this construct and tTA expression plasmid pUHD15-1 were co-transfected into cells, in the absence or presence of the effector substance  
15 doxycycline, changes in luciferase activity would indicate the ability of tTA system to regulate the transcription of target gene transcription. To test whether a tetracycline-regulated gene expression system functions in primary rat cardiomyocytes, cell cultures were co-transfected with tTA expression plasmid pUHD15-1 (0.25 $\mu$ g) and tTA-stimulated luciferase gene expression construct pUHC13-3 (0.25 $\mu$ g). As a control, the same amounts  
20 of reporter plasmid pUHC13-3 plus an empty vector, pUHD10-3, were transfected into cardiomyocytes from same cell preparation. After transfection, luciferase activities in cell extracts were examined at various time points, using the luciferase assay as described in Example 3. In the absence of tTA, only very low levels of reporter gene activity were detected during the 48 hours post-transfection. tTA expression resulted in a dramatic  
25 increase in reporter activity during this period, and target gene activities reached a maximum at 12 hours after transfection, as shown in Figure 11A. At 12 hours post-transfection, the luciferase activities in the tTA-transfected cells were over 30-fold higher than those in the cell without tTA transfection (Figure 11A and 11B). When the cells were treated with 1  $\mu$ g/ml doxycycline (Fluka Chemical Corp.) after transfection, transactivation  
30 of the target gene was markedly repressed. However, the doxycycline treatment itself caused a transiently low level expression of the target gene (about a 4-fold increase at 12 hour post-transfection) (Figures 11A and 11B), suggesting that doxycycline repression to



the target gene in primary cardiomyocytes is not complete. It is possible that exogenous gene expression in transient transfection is not under tight control of native gene regulation machinery in these cells. Doxycycline treatment might cause a weak signal to stimulate the low level expression of this target gene. Therefore, doxycycline does not appear to be the best effector to regulate cardiac gene transcription in this system.

***Transcription of the hypertrophy-sensitive genes induced by tTA-regulated calcineurin expression system depends on calcineurin gene dose***

Constitutively expressed active calcineurin activates the transcription of ANF reporter gene in primary rat cardiomyocytes. Furthermore, the transcription of cardiac  $\alpha$ -actin reporter gene is induced by tTA-stimulated active calcineurin expression in Pac-1 smooth muscle cells. To detect the effects of active calcineurin on the cardiac gene transcription in primary cardiomyocytes and determine the optimal concentration of calcineurin construct in transfection to induce cardiac gene expression, various amounts of tTA-dependent activated calcineurin expression construct p10-3CaN, 0.25  $\mu$ g tTA expression plasmid pUHD15-1 and 0.25  $\mu$ g cardiac  $\alpha$ -actin promoter-reporter plasmid CardA-Luc were transfected into primary rat cardiomyocytes. The results are shown in Figure 12. The empty vector, pUHD10-3, was used as a control to substitute for p10-3CaN or to complement total DNA to 0.75  $\mu$ g/per well. At 24 hours after transfection, the expression levels of cardiac  $\alpha$ -actin reporter gene were analyzed by the luciferase assay, as described in Example 3. As shown in Figure 12, in the absence of exogenous active calcineurin, the level of reporter gene expression was quite low, whereas the transcription of cardiac  $\alpha$ -actin reporter gene was gradually enhanced by increasing doses of transfected p10-3CaN. When the amount of calcineurin DNA was up to 0.25  $\mu$ g, the expression of reporter gene was increased to nearly maximum levels. Clearly, the expression of cardiac  $\alpha$ -actin gene is active calcineurin gene dose-dependent. 0.25  $\mu$ g p10-3CaN was used as the optimal dose in subsequent experiments.

***Induced expression of an active form of calcineurin rapidly activates the transcription of three hypertrophy-sensitive genes in myocardial cells***

To further assess the ability of calcineurin to regulate the cardiac gene expression in myocardial cells, we studied the time courses of expression of three hypertrophic response genes under the induction of active calcineurin expression. Cardiac  $\alpha$ -actin,

skeletal  $\alpha$ -actin, and ANF promoter-Luc plasmids were used as reporters. Primary neonatal rat cardiomyocytes were co-transfected with either reporter, pUHD15-1, and p10-3CaN, or empty vector pUHD10-3 instead of p10-3CaN, together with pUHD15-1 and reporter plasmids. The amount of each plasmid was 0.25  $\mu$ g. Transfections were carried out as described in Example 3. At various time points after transfection, the luciferase activities of cell extracts were measured, as described in Example 3. In each experiment, the highest level of luciferase activity was used as the maximum response of reporter gene, all data were normalized to a percentage of the maximum response. In the absence of the expression of exogenous active calcineurin, the transcription levels of three cardiac genes were very low. However, active calcineurin expression markedly induced the transcription of three cardiac genes, as shown in Figure 13. The expression of cardiac  $\alpha$ -actin, skeletal  $\alpha$ -actin, and ANF genes were all significantly increased to nearly maximum levels 48 hours post-transfection, suggesting that active calcineurin rapidly activates the transcription of these hypertrophy response gene in primary cardiomyocytes.

When the same cardiac cell preparations were co-transfected with same amounts of reporter, tTA, and calcineurin plasmid DNA as indicated in Figure 14, expression levels of three cardiac genes were detected at 48 hours after transfection. Compared with the controls in which only empty vector pUHD10-3 instead of calcineurin plasmids, together with reporter and tTA plasmid, were transfected into the cardiomyocytes, calcineurin strongly activated the transcription of cardiac  $\alpha$ -actin by about 8 to 10-fold, and moderately activated ANF and skeletal  $\alpha$ -actin expression by about 6-fold and 3-fold, as shown in Figures 14B and 14C.

These results not only indicated the stimulatory effect of calcineurin on the transcription of hypertrophy-sensitive genes, but also demonstrated utility of the inducible gene expression system. The ability of activated calcineurin to induce the transcription of hypertrophy response gene in the cardiomyocytes is consistent with the result from transgenic mice overexpressing the constitutively active form of calcineurin (Molkentin et al., 1998). For example, the mRNA level of skeletal  $\alpha$ -actin gene in the heart of calcineurin transgenic mice was about 5-fold higher than that in normal mouse heart. The increased fold of ANF gene expression induced by calcineurin in our inducible expression system is similar to that of BNP gene activation in calcineurin transgenic mice. However,

the increased fold in transcription level of cardiac  $\alpha$ -actin gene in our system is higher than that in transgenic mice overexpressing calcineurin.

***Repression of calcineurin induction of hypertrophic response gene expression by cyclosporin A***

5 To evaluate whether the transcriptional activation of hypertrophy-sensitive genes by activated calcineurin could be inhibited by CsA, a specific inhibitor of calcineurin, both the myocardial cell cultures transfected with either p10-3CaN construct or empty vector pUND10-3, together with tTA and cardA-Luc reporter plasmids as described in Example 3, were treated with different concentrations of CsA (Sandoz Pharmaceuticals Corp., East  
10 Hanover, NJ) or without CsA treatment from 0 hour after transfection. At various time points, the luciferase activities in those cells were assayed and compared. In the control group which had no calcineurin transfection, CsA treatment only resulted in a less decrease in the transcription level of cardiac  $\alpha$ -actin gene compared with no treatment, as shown in Figure 15. However, cardiac  $\alpha$ -actin gene expression stimulated by activated  
15 calcineurin was significantly repressed by all those different concentrations of CsA, as shown in Figure 15, further supporting the conclusion that the expression of these cardiac hypertrophic response gene is activated by a calcineurin-dependent signal transduction pathway. At 72 hours after transfection, cardiac  $\alpha$ -actin gene activity induced by calcineurin was inhibited over 80% by 100nM or 150nM CsA treatment, as shown in  
20 Figure 15, indicating that the very low concentrations of CsA can inhibit transcriptional activation of cardiac  $\alpha$ -actin gene induced by calcineurin. Therefore, 100nM was chosen as the optimal concentration of CsA in subsequent experiments.

To further demonstrate the effect of CsA on the transcription of hypertrophy-sensitive genes induced by calcineurin, we analyzed how the expression of cardiac  $\alpha$ -actin  
25 promoter-reporter gene under active calcineurin stimulation responds to CsA. Primary cardiomyocytes were co-transfected with either tTA, cardiac  $\alpha$ -actin reporter constructs, and calcineurin, or tTA, cardiac  $\alpha$ -actin reporter gene and empty vector as the control. CsA treatment (100 nM) was initiated at 0 hour after transfection. Cardiac  $\alpha$ -actin gene activity in cardiac cell cultures was examined by luciferase assay and results were  
30 normalized to a percentage of the maximum response. In the cells transfected with the empty vector, the expression of cardiac  $\alpha$ -actin gene was low and there was no marked

different in the gene activity between the CsA treated and untreated cells, as shown in Figure 16. However, CsA treatment lead to a dramatic reduction in cardiac gene transcription in active calcineurin-transfected cells. 48 hours after CsA treatment, the expression of cardiac  $\alpha$ -actin gene activated by calcineurin was decreased nearly to basal levels, as shown in Figure 16.

Based on the results above, we tested the effects of CsA on the calcineurin-mediated transcriptional activation of ANF, cardiac  $\alpha$ -actin and skeletal  $\alpha$ -actin genes. Equal amounts of each reporter construct, together with tTA and p10-3CaN DNA, were co-transfected into the cells derived from the same primary culture preparation. The empty vector, pUHD10-3, instead of p10-3CaN was used as a control. After transfection, the cells transfected with active calcineurin were cultured for 48 hours in either culture medium containing 100nM CsA or in culture medium lacking CsA. Cells transfected with empty vector were cultured in medium lacking CsA for same amount of time. Then these cardiomyocytes were lysed to analyze the activities of these cardiac hypertrophy response genes. The results are shown in Figure 17. The transcriptional activation of ANF and skeletal  $\alpha$ -actin genes mediated by active calcineurin was almost completely blocked by CsA in primary rat cardiomyocytes: about 80% of transcriptional activity of cardiac  $\alpha$ -actin gene up-regulated by the action of calcineurin was inhibited by CsA.

*Repression of the transcription of hypertrophy-sensitive genes by CsA treatment is reversible*

Due to practical requirements in complex studies, the ability to bidirectionally regulate the expression of exogenous gene would have a great significance in the studies of gene function as well as in gene therapies. To evaluate whether the repressed expression of the exogenous hypertrophic response genes in our system could be reversibly controlled by discontinuing CsA treatment, primary cardiomyocytes from the same preparation were transfected with tTA, calcineurin construct, and cardiac  $\alpha$ -actin reporter plasmids. After transfection, all cells were treated with 100nM CsA. 24 hours later, a portion of the cells were continuously cultured in CsA-containing medium, while another portion of the cells were switched to medium without CsA. At various time points, reporter gene expression in these cardiomyocytes was examined by luciferase assay. As shown in Figure 18, during a 72-hour period, the reporter gene activities in CsA-treated cells were low. However, the expression of the cardiac gene was rapidly elevated after discontinuing CsA treatment. 48

hours after removing CsA from media (72 hour time point), cardiac  $\alpha$ -actin reporter gene activity had increased about 2.5-fold compared with that in cells treated with CsA. The data demonstrated that the repressed transcription of the cardiac gene by CsA could be rapidly reversed. Therefore, based on these results shown above, the expression of exogenous hypertrophy-sensitive genes in primary cardiomyocytes, under the experimental conditions used, could be rapidly regulated in either a positive or a negative way.

#### EXAMPLE 5

##### *Characterization of effects of CaMKIV and YY1 on expression of hypertrophy-sensitive genes in a cultured cardiomyocyte cell model*

Experimental procedures with respect to cardiomyocyte cell culture, cardiomyocyte transient transfections, and luciferase activity measurements were carried out as described in Example 3. Cardiac hypertrophy-sensitive promoter-reporter constructs are as described in Example 3.

#### Results

##### Constitutively active CaMKIV increases transcription of cardiac hypertrophy-sensitive gene promoter-reporter activity in neonate cardiomyocytes

A cDNA encoding a constitutively active form of CaMKIV, as described above, was inserted into an expression vector containing the  $\alpha$ -MHC promoter. Neonate cardiomyocytes were co-transfected with skeletal  $\alpha$ -actin-luciferase plasmid described in Example 3 and a constitutively active mutant form of CaMKIV in an expression plasmid. pSG (empty vector) served as a negative control. Luciferase activity was measured at various times after transfection. The results are shown in Figure 19. Overexpression of constitutively active exogenous CaMKIV increased skeletal  $\alpha$ -actin promoter activity more than 300% over controls.

The effect of overexpression of constitutively active exogenous CaMKIV on a cardiac  $\alpha$ -actin promoter-reporter construct in neonate cardiomyocytes was explored. Cardiomyocytes were co-transfected with a cardiac  $\alpha$ -actin promoter-reporter construct as described in Example 3, and various amounts of the expression plasmid encoding constitutively active CaMKIV. The results, shown in Figure 20, indicate that overexpression of constitutively active CaMKIV results in modest increases in cardiac  $\alpha$ -actin promoter activity. Peak induction (about 7-fold over control) was achieved using 0.01  $\mu$ g

CaMKIV expression plasmid, and dropped to nearly control levels when 0.10  $\mu$ g CaMKIV expression plasmid was used.

In contrast, when a similar dose-dependence study was performed using skeletal  $\alpha$ -actin promoter-reporter plasmid, over-expression of constitutively active CaMKIV  
5 resulted in substantial increases in skeletal  $\alpha$ -actin promoter activity, as shown in Figure 21. A similar result was observed using an ANF promoter-reporter construct, as shown in Figure 22.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent  
10 application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the  
15 invention, which is delineated by the appended claims.

## CLAIMS

1. A method of treating a cardiac hypertrophy-induced dysfunction, comprising administering to a subject in need thereof an amount of a composition effective to  
5 diminish or reverse progression of the dysfunction, wherein the composition inhibits activity of protein selected from the group consisting of calcineurin, calcium calmodulin dependent kinase IV (CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ), YY1, p38 and MKK6 or a derivative thereof.
- 10 2. The method according to claim 1, wherein the composition comprises a substance selected from the group consisting of a cyclosporin, FK506, and derivatives thereof that inhibit activity of the protein or derivative thereof.
- 15 3. The method according to claim 1, wherein the composition modulates activity of at least one gene or gene product that is expressed in cardiomyocytes in response to a hypertrophic signal.
- 20 4. The method according to claim 3, wherein the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal is selected from the group consisting of  $\beta$ -myosin heavy chain,  $\alpha$ -skeletal actin, cardiac actin, atrial natriuretic factor, and b-type natriuretic factor.
- 25 5. The method according to claim 1, wherein the composition reduces the activity of calcineurin.
6. The method according to claim 5, wherein the composition comprises an inhibitor of calcineurin phosphatase activity.
- 30 7. The method according to claim 1, wherein the substance reduces the activity of calcium calmodulin dependent kinase IV (CaMKIV).

8. The method according to claim 7, wherein the substance is an inhibitor of CaMKIV kinase activity.
9. The method according to claim 7, wherein the substance reduces expression levels of a gene encoding CaMKIV.
10. The method according to claim 1, wherein the substance increases the activity of calcium calmodulin dependent kinase II alpha (CaMKII $\alpha$ ).
11. The method according to claim 10, wherein the substance is an activator of CaMKII $\alpha$  kinase activity.
12. The method according to claim 11, wherein the substance increases expression levels of a gene encoding CaMKII $\alpha$ .
13. The method according to claim 1, wherein the substance increases the activity of YY1.
14. The method according to claim 13, wherein the substance reduces the activity p38.
15. The method according to claim 1, wherein the substance inhibits p38 kinase activity.
16. The method according to claim 15, wherein the substance reduces expression levels of a gene encoding p38.
17. The method according to claim 1, wherein the substance reduces the activity MKK6.
18. The method according to claim 17, wherein the substance inhibits MKK6 kinase activity.
19. The method according to claim 18, wherein the substance reduces expression levels of a gene encoding MKK6.



20. The method according to claim 1, wherein the composition has therapeutic activity toward cardiac hypertrophy.

21. The method according to claim 1, wherein the composition further comprises a pharmaceutically acceptable excipient.

22. A method of screening substances suitable for use in treating a cardiac hypertrophy-induced disease state, comprising:

- a) contacting a substantially isolated enzyme with the substance, wherein the enzyme is involved in modulating levels of active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal;
- b) measuring an activity of the enzyme in the presence of the substance; and
- c) providing a control by measuring the activity of the enzyme in the absence of the substance,

wherein modulation of the activity of the enzyme by the substance as compared to the control indicates that the substance is suitable for use in treating the cardiac hypertrophy-induced cardiac disease state.

23. The method according to claim 22, wherein the substantially isolated enzyme is calcineurin, calcium calmodulin dependent kinase IV (CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ), YY1, p38 and MKK6 or a derivative thereof.

24. The method according to claim 22, wherein the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal is selected from the group consisting of  $\beta$ -myosin heavy chain,  $\alpha$ -skeletal actin, atrial natriuretic factor, cardiac actin, and b-type natriuretic factor.

25. A method of screening substances for use treating a cardiac hypertrophy-induced disease state, comprising:

- a) contacting a first substantially isolated cardiomyocyte with a substance;

- b) measuring a cardiomyocyte parameter associated with hypertrophy of the first cardiomyocyte in the presence of the substance; and
- c) providing a control by measuring the cardiomyocyte parameter associated with hypertrophy in a second substantially isolated cardiomyocyte not contacted with the substance,

5

wherein modulation of the cardiomyocyte parameter by the substance as compared to the control indicates that the substance is suitable for use in treating the cardiac hypertrophy-induced cardiac disease state.

10

26. The method according to claim 25, wherein the first and second cardiomyocytes are primary cardiomyocytes.

15

27. The method according to claim 26, wherein the first and second cardiomyocytes are substantially isolated from a transgenic non-human animal, and wherein the transgenic animal comprises a transgene which is expressed in cardiomyocytes and wherein an expression product of the transgene modulates levels of active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

20

28. The method according to claim 26, wherein the expression product of the transgene is calcineurin, calcium calmodulin dependent kinase IV (CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ), YY1, p38 and MKK6 or a derivative thereof.

25

29. The method according to claim 26, wherein the cardiomyocyte parameter being measured is expression levels of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

30

30. The method according to claim 29, wherein the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal encodes a protein selected from the group consisting of  $\beta$ -myosin heavy chain,  $\alpha$ -skeletal actin, atrial natriuretic factor, cardiac actin, and b-type natriuretic factor.

31. The method according to claim 25, wherein the cardiomyocyte parameter being measured is cell diameter.

32. The method according to claim 25, wherein the cardiomyocyte parameter being measured is cell mass.

33. The method according to claim 25, wherein the wherein the cardiomyocyte parameter being measured is levels of active product of at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal.

34. The method according to claim 33, wherein the at least one gene that is expressed in cardiomyocytes in response to a hypertrophic signal encodes a protein selected from the group consisting of  $\beta$ -myosin heavy chain,  $\alpha$ -skeletal actin, atrial natriuretic factor, cardiac actin, and b-type natriuretic factor.

35. A method of screening substances for use in treating a cardiac hypertrophy-induced disease state, comprising:

a) administering a substance to a non-human transgenic animal, or an in vitro part thereof, having a genome comprising a transgene comprising a coding region encoding a calcineurin, calcium calmodulin dependent kinase IV (CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ), YY1, p38 and MKK6 or a derivative thereof and a cardiomyocyte-specific transcriptional regulatory element,;

b) measuring a cardiac parameter of the animal or part thereof in the presence of the substance; and

c) providing a control by measuring the cardiac parameter in a corresponding animal or part thereof not administered with the substance,

wherein modulation of the cardiac parameter by the substance as compared to the control indicates that the substance is suitable for use in treating the cardiac hypertrophy-induced cardiac disease state.

36. The method according to claim 35, wherein the cardiac parameter being measured is selected from the group consisting of ventricular mass, dilated cardiac myopathy, fibroid

deposition, cardiomyocyte organization, calcium ion flux, stroke length, and ventricular output, and dP/dT.

37. A transgenic non-human animal comprising a transgene which comprises:

5 a) a coding region which encodes a gene product which modulates transcription of at least one cardiac hypertrophy-sensitive gene; and

b) a transcriptional control element (TRE) operably linked to said coding region, wherein said TRE comprises a cardiomyocyte-specific promoter,

10 wherein said transgenic animal is characterized in that it has a substantially increased probability of spontaneously developing cardiac hypertrophy when compared to a non-transgenic littermate.

38. The transgenic animal according to claim 37, wherein the at least one cardiac hypertrophy-sensitive gene is selected from the group consisting of an atrial natriuretic factor gene, a b-type natriuretic peptide gene, a  $\beta$ -myosin heavy chain gene, and an  $\alpha$ -skeletal actin gene.

39. The transgenic animal according to claim 37, wherein the cardiomyocyte-specific promoter is an  $\alpha$ -myosin heavy chain promoter.

40. The transgenic animal according to claim 37, wherein said TRE further comprises an inducible promoter element.

41. The transgenic animal according to claim 40, wherein the inducible promoter element is regulated by a tetracycline transactivator.

42. The transgenic animal according to claim 37, wherein the coding region encodes calcineurin, calcium calmodulin dependent kinase IV (CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ), YY1, p38 and MKK6 or a derivative thereof.

43. The transgenic animal according to claim 42, wherein a phosphatase activity of the calcineurin is constitutively active.

44. A transgenic non-human animal comprising a transgene which comprises:

5 a) a coding region which encodes a gene product which modulates transcription of at least one cardiac hypertrophy-sensitive gene; and

b) a transcriptional control element (TRE) operably linked to said coding region, wherein said TRE comprises a cardiomyocyte-specific promoter,

wherein said transgenic animal is characterized in that it has a substantially decreased  
10 probability of spontaneously developing cardiac hypertrophy when compared to a non-transgenic littermate.

45. The transgenic animal according to claim 44, wherein the coding region encodes a dominant negative mutant of calcineurin.

46. The transgenic animal according to claim 45, wherein the mutant substantially lacks phosphatase activity, and wherein the mutant retains calmodulin binding.

47. A method of making a transgenic mouse animal model of cardiac hypertrophy  
20 comprising:

a) introducing into a mouse embryo a polynucleotide comprising a coding region which encodes a gene product or an enzymatically active variant thereof selected from the group consisting of calcineurin, calcium calmodulin dependent kinase IV

(CaMKIV), calcium calmodulin dependent kinase II alpha (A and B) (CaMKII $\alpha$ ),

25 YY1, p38 and MKK6, said coding region being operably linked to a transcriptional regulatory element comprising a cardiomyocyte-specific promoter; and

b) transferring the embryo into a foster mother mouse,

wherein said transgenic mouse is characterized in that it has a substantially increased  
30 probability of spontaneously developing cardiac hypertrophy when compared to a non-transgenic littermate.

48. A transgenic mouse produced by breeding mice obtained according to claim 47.

49. The mice obtained according to claim 48, wherein the progeny contain at least two of the gene products.

5 50. A cell derived from the transgenic animal of claim 37.

51. The cell according to claim 50, wherein the cell is a cardiomyocyte.

52. A cell derived from the transgenic animal of claim 44.

10

53. The cell according to claim 51, wherein the cell is a cardiomyocyte.

54. A method of screening a substance for the ability to decrease reduce cardiac hypertrophy, comprising:

15

- a) administering the substance to the transgenic animal of claim 37;
- b) measuring a cardiac parameter or function in the animal compared to a transgenic littermate not administered with the substance.

FIGURE 1

# Nuclear Signaling and Transcriptional Control of Hypertrophy Induced Embryonic Gene Expression

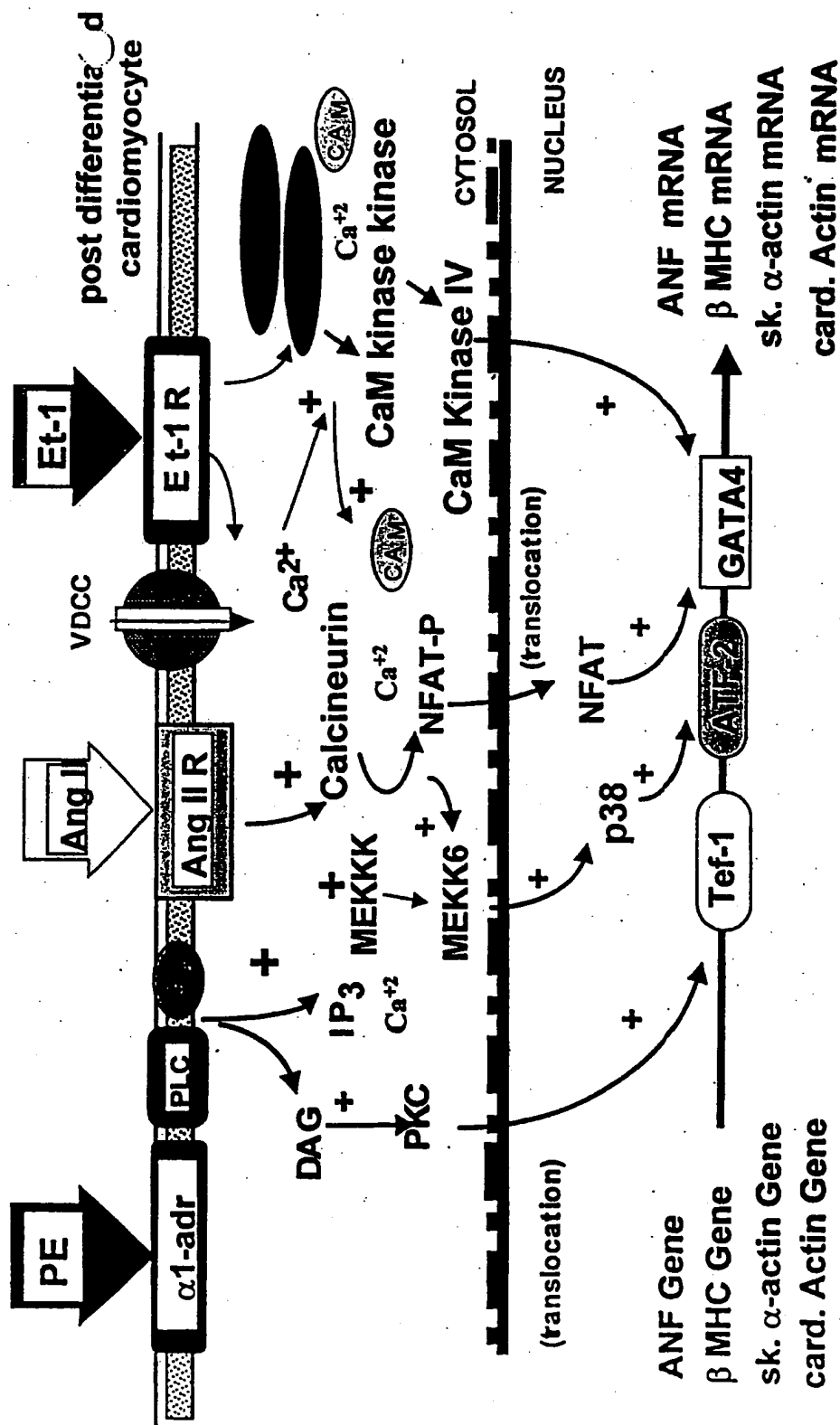
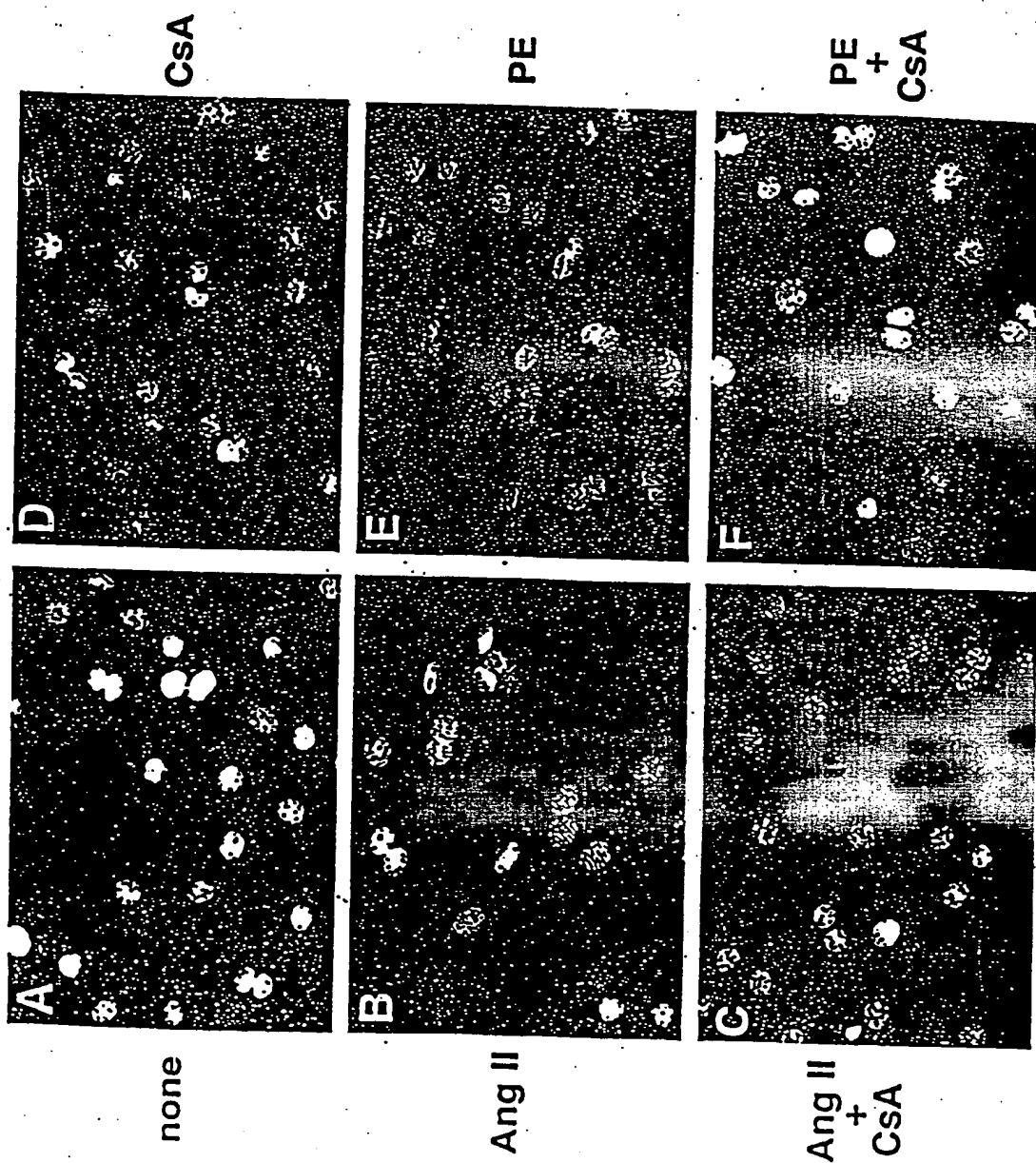
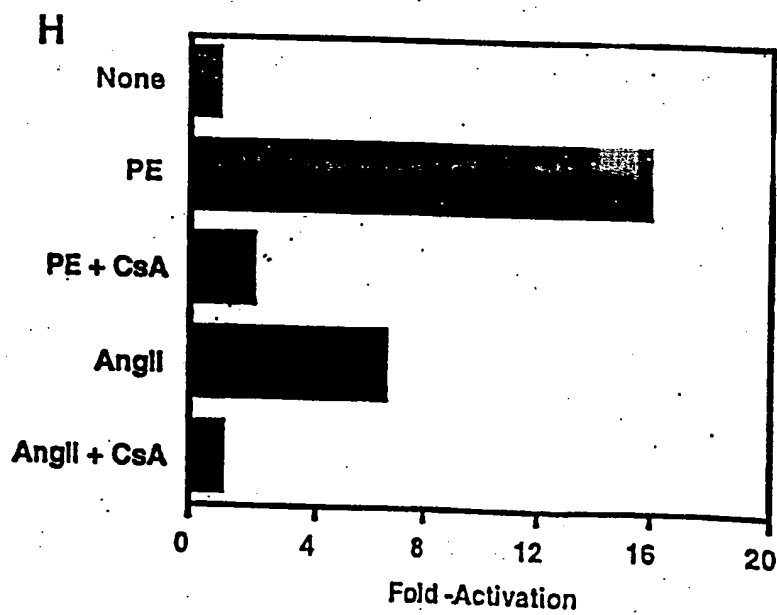
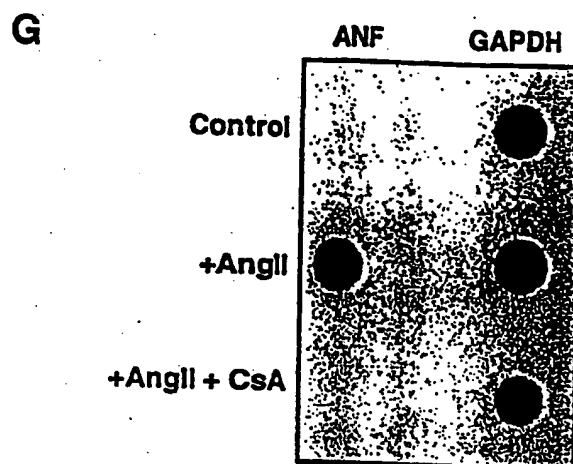


FIGURE 2 A-F





**FIGURE 2 G-H**

**FIGURE 3**

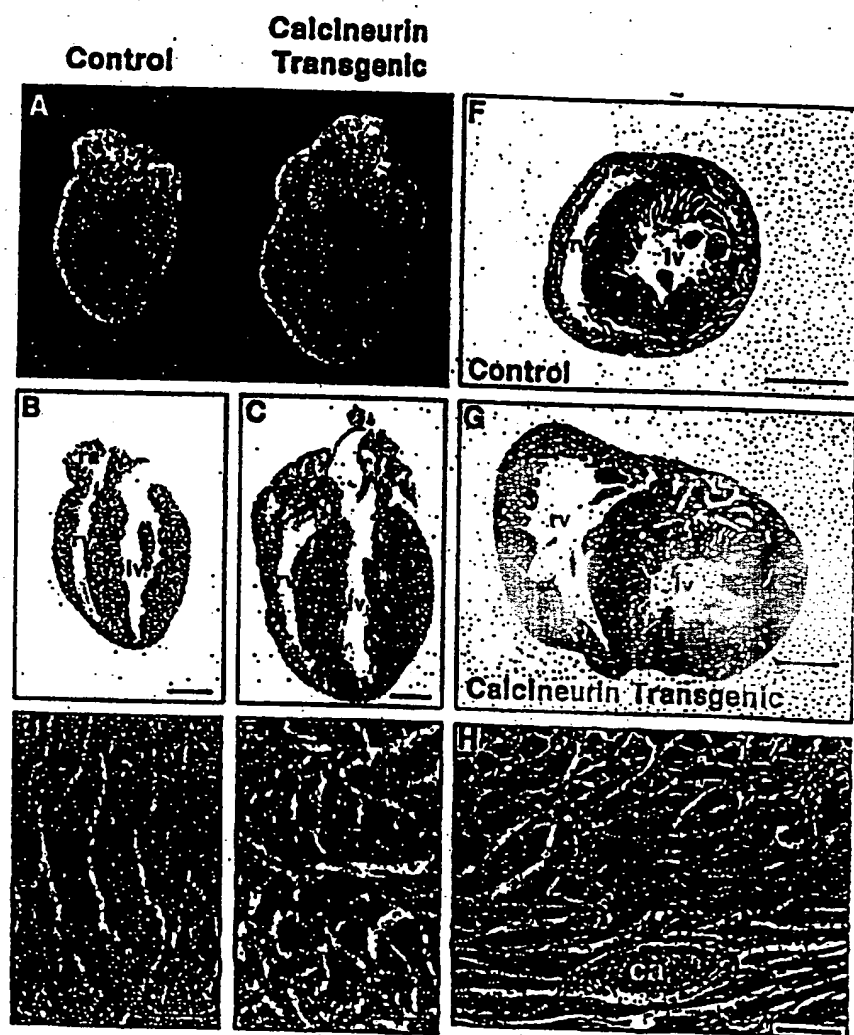
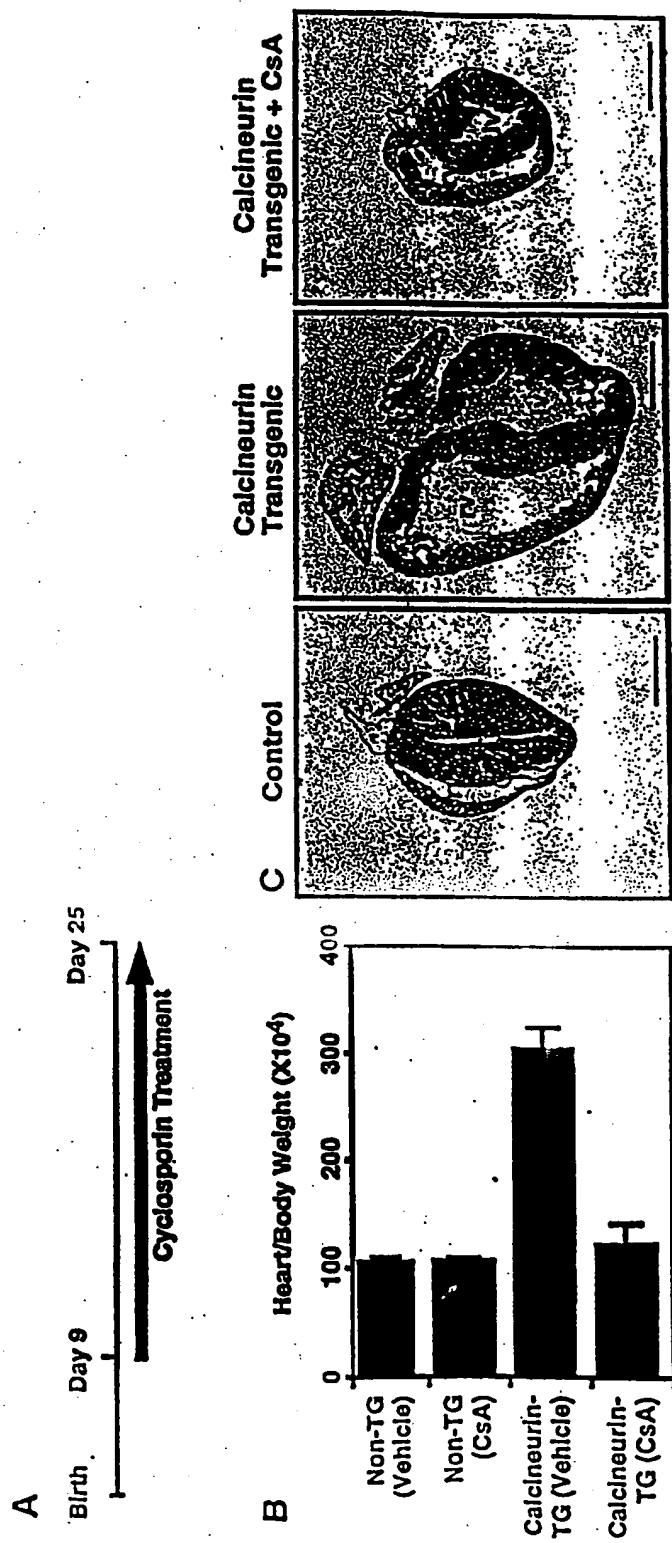


FIGURE 4



**FIGURE 5**

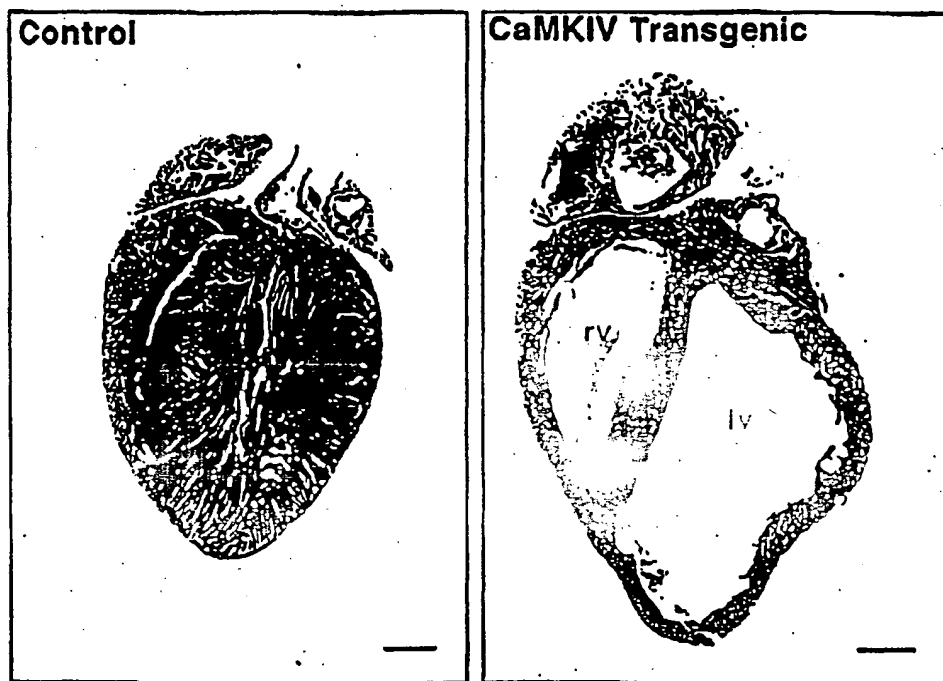


FIGURE 6

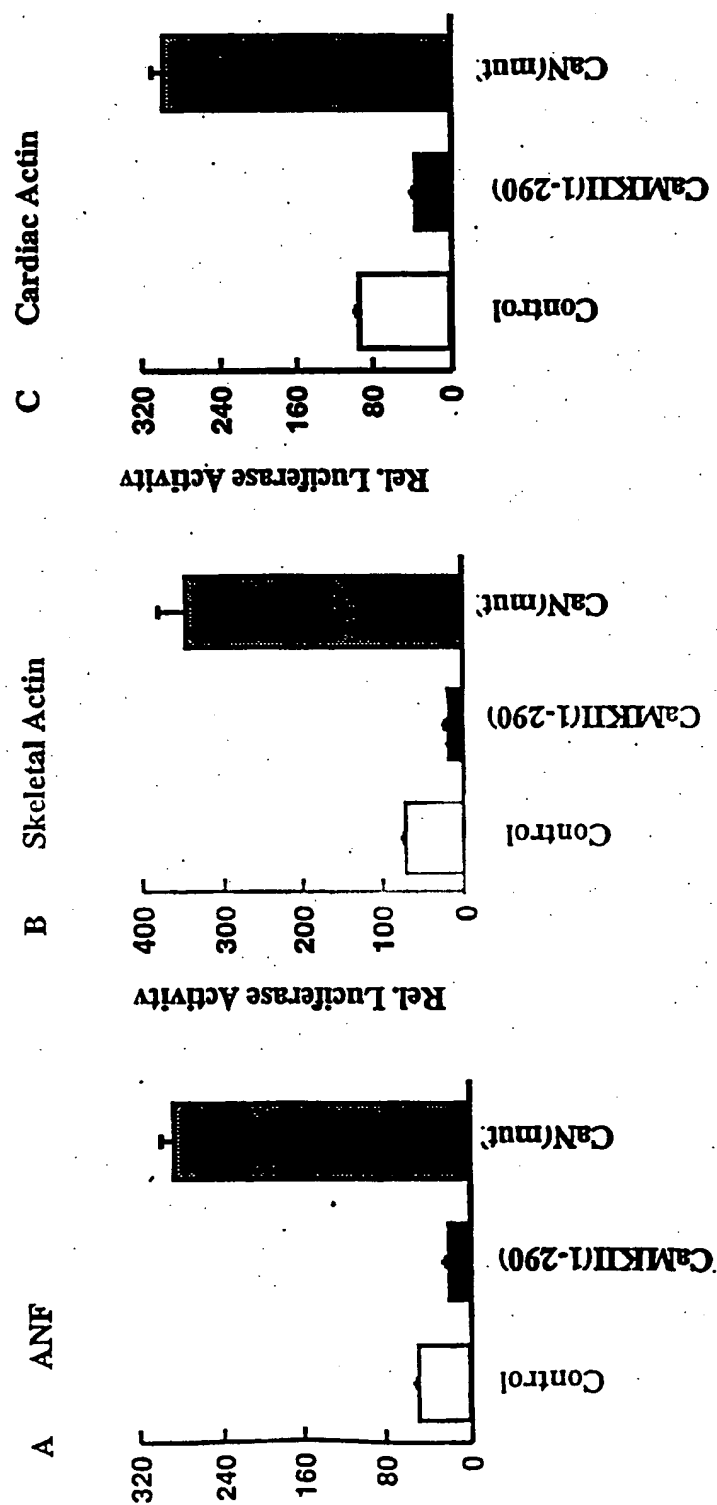


FIGURE 7

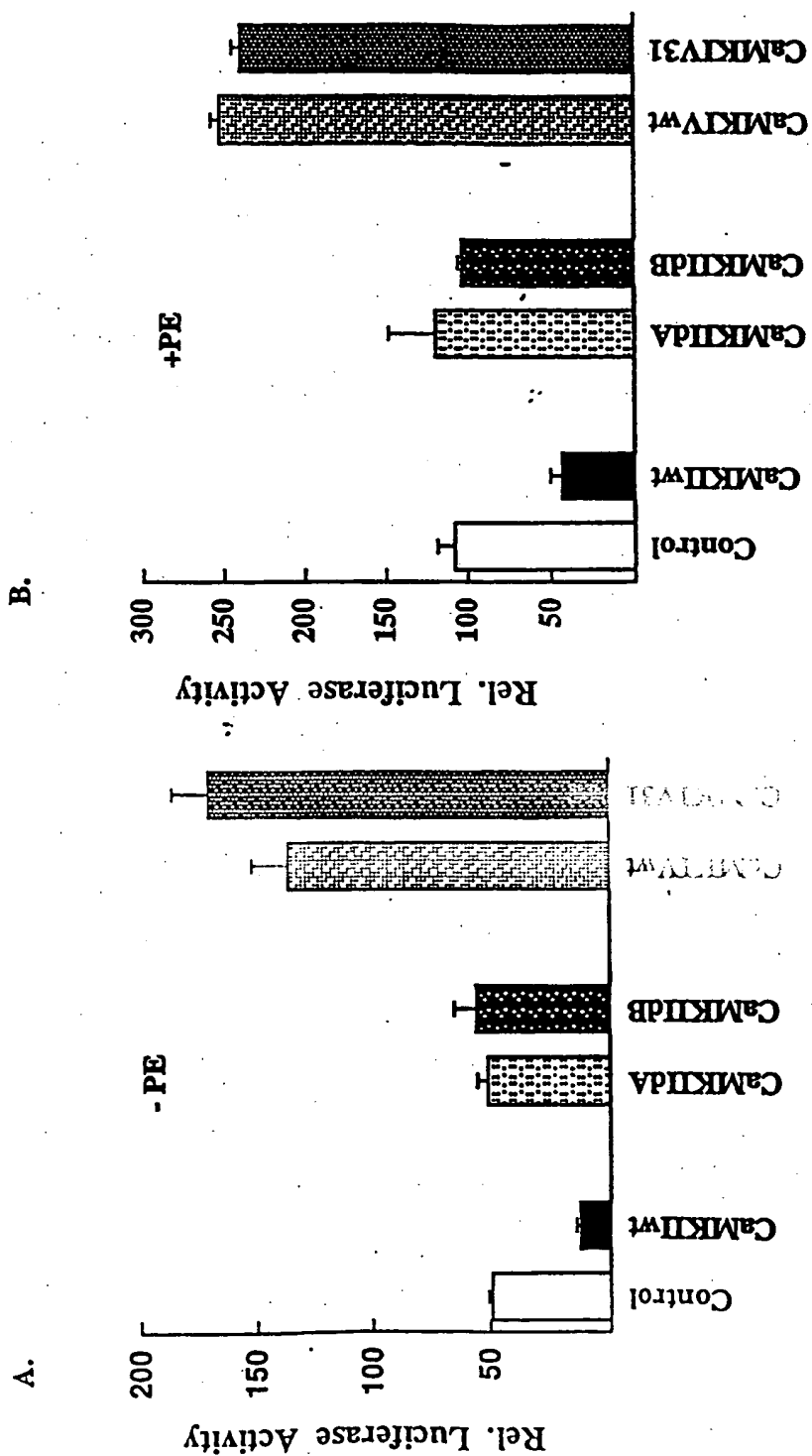
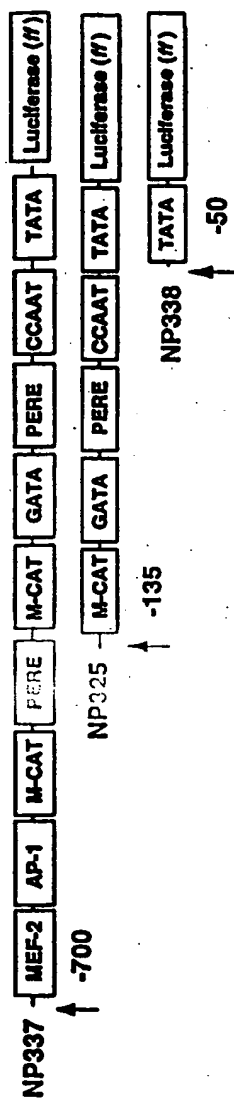
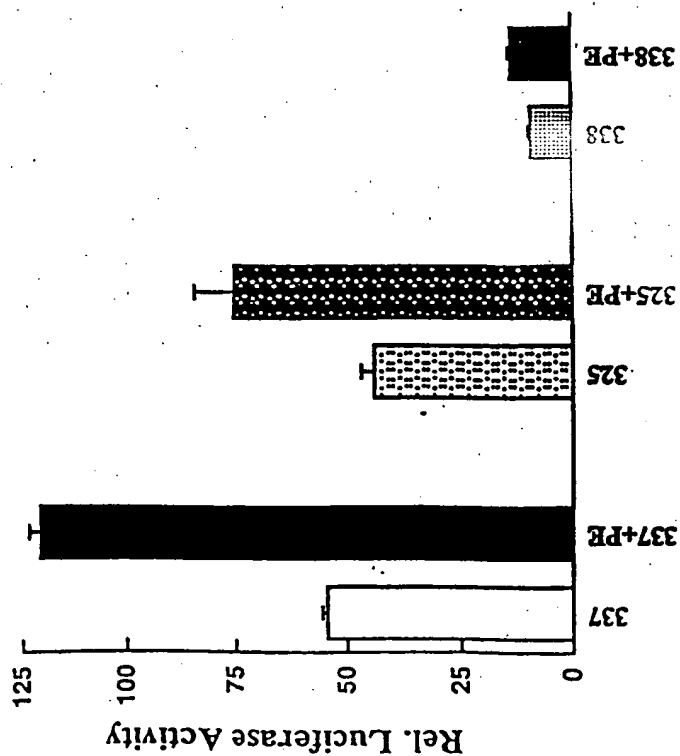


FIGURE 8

A.



B.



C.

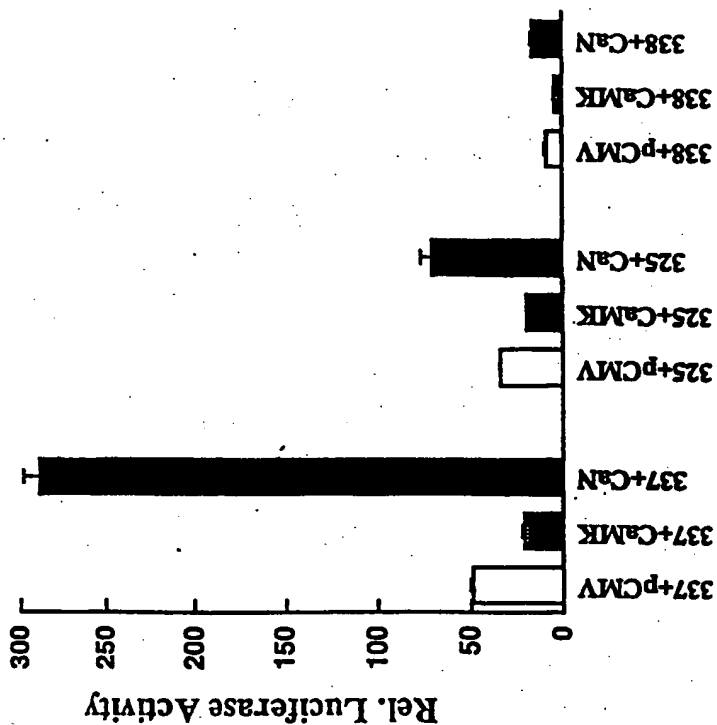
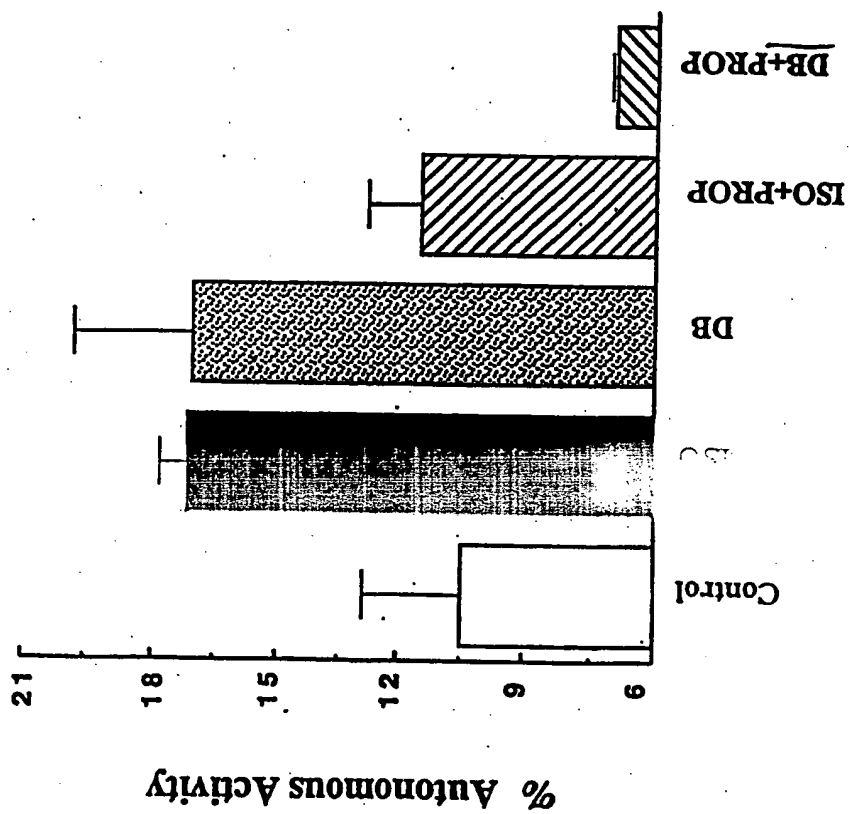
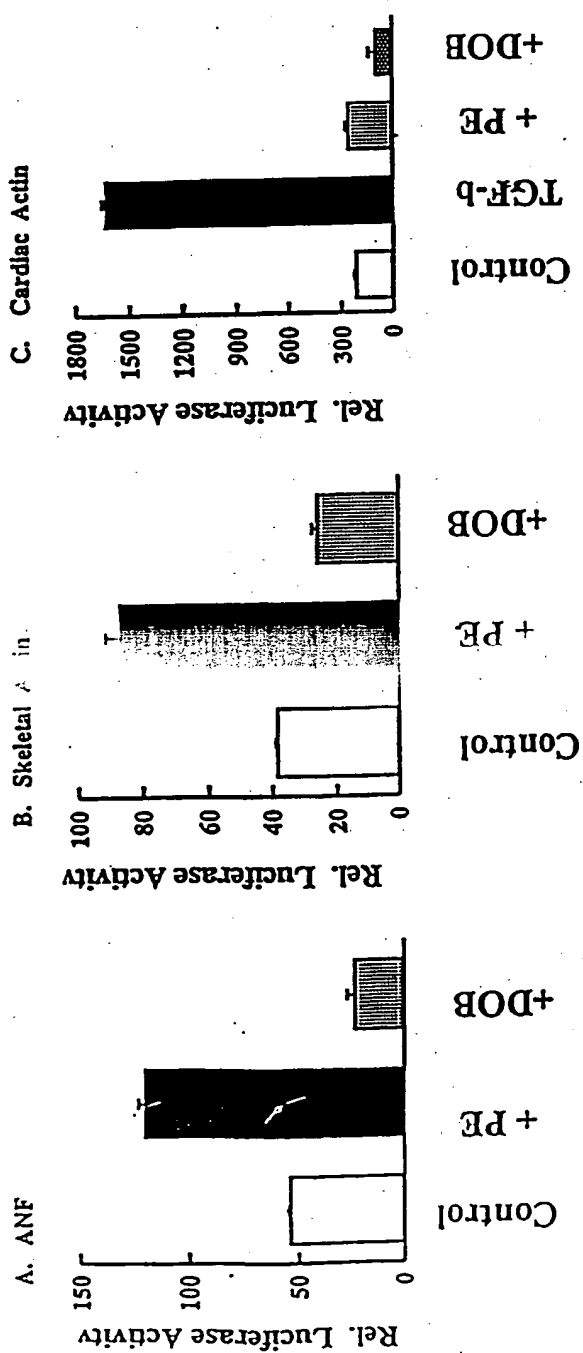


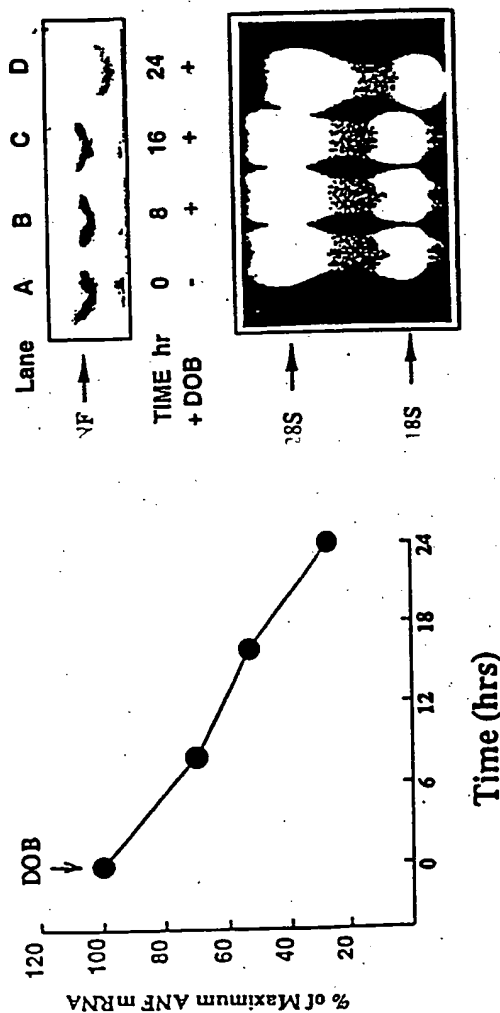
FIGURE 9



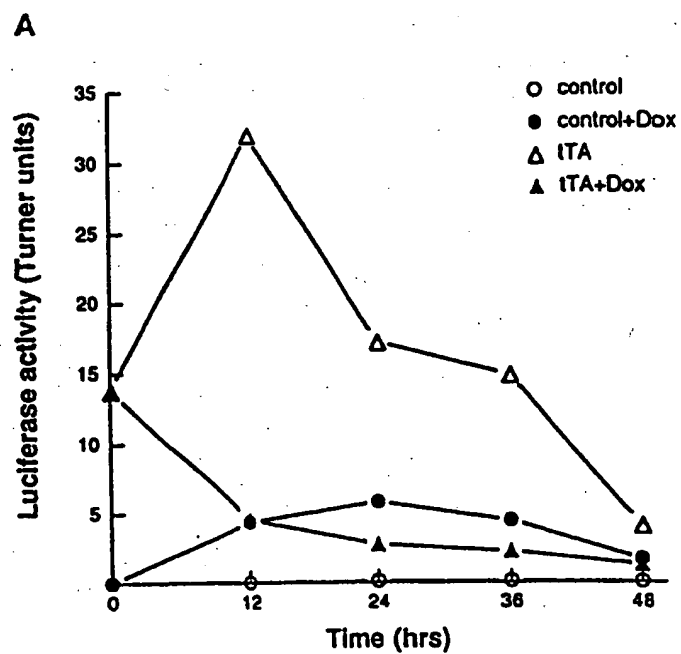


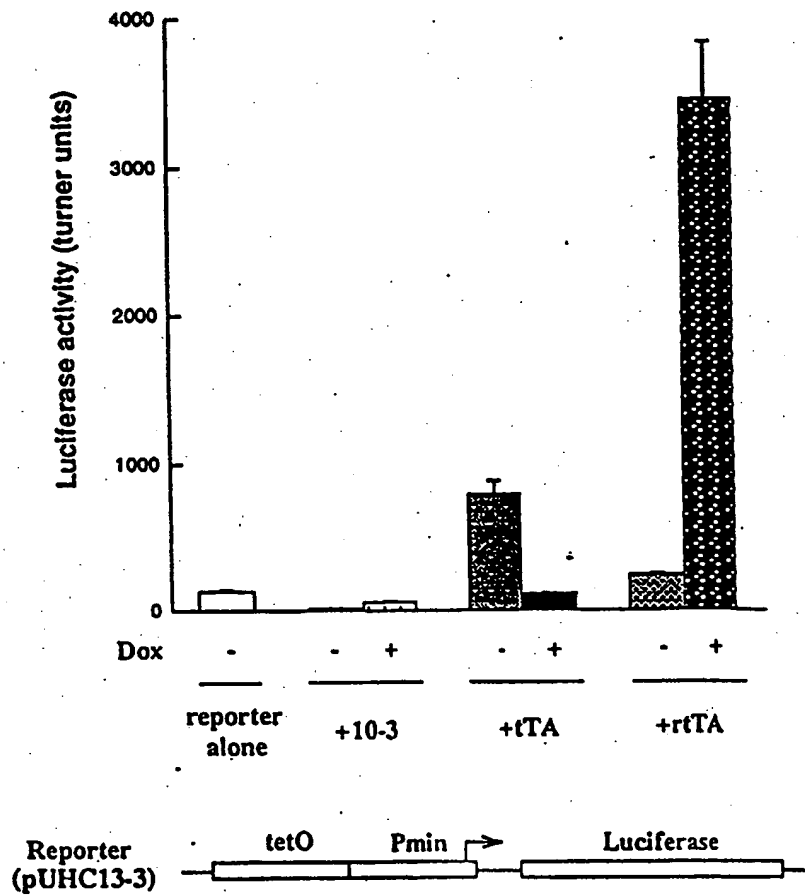


D.



**FIGURE 10**

**FIGURE 11A**

**FIGURE 11B**

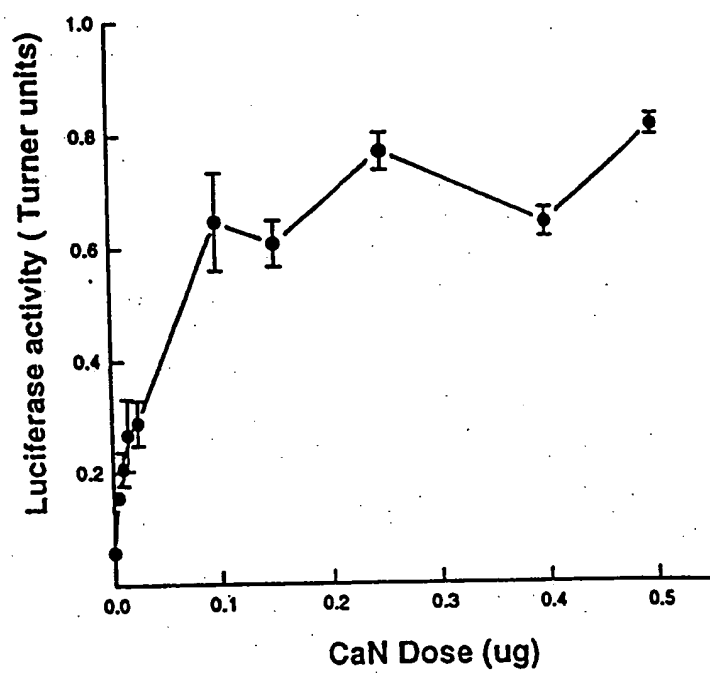
**FIGURE 12**

FIGURE 13

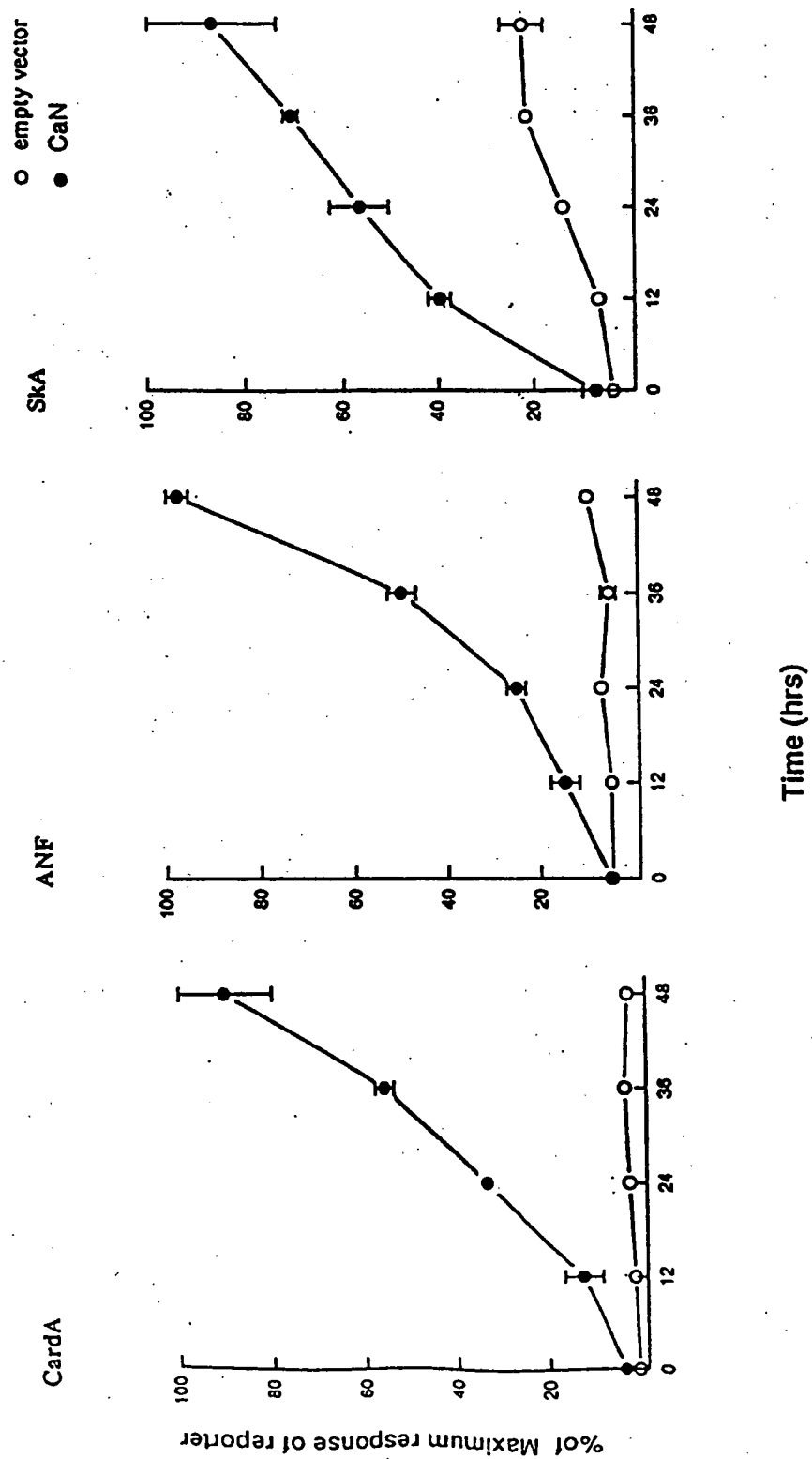
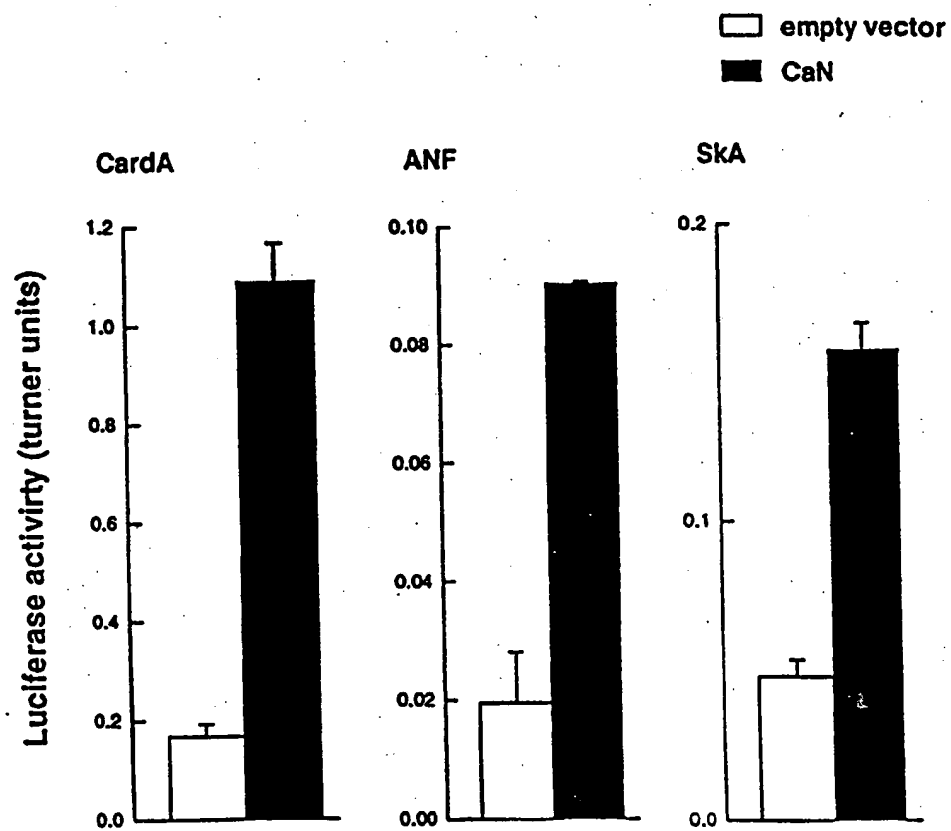
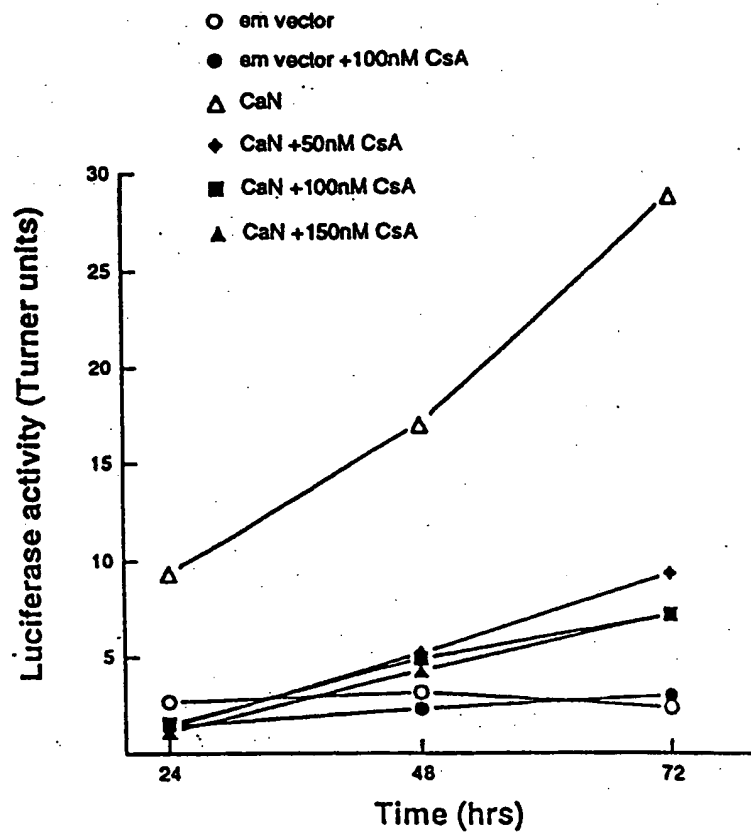
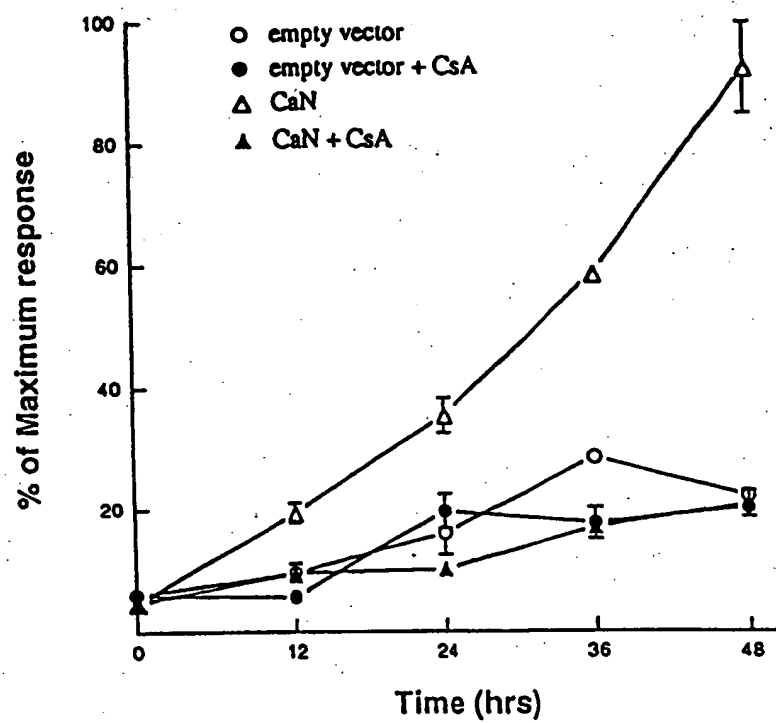


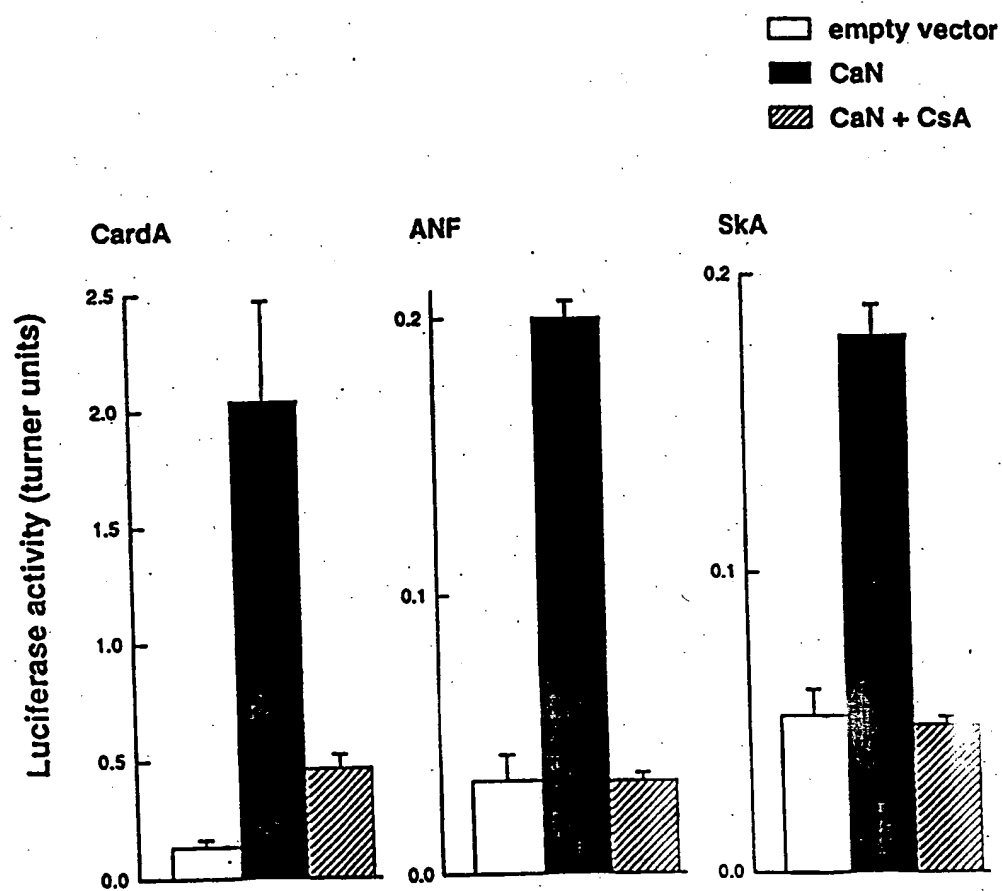
FIGURE 14

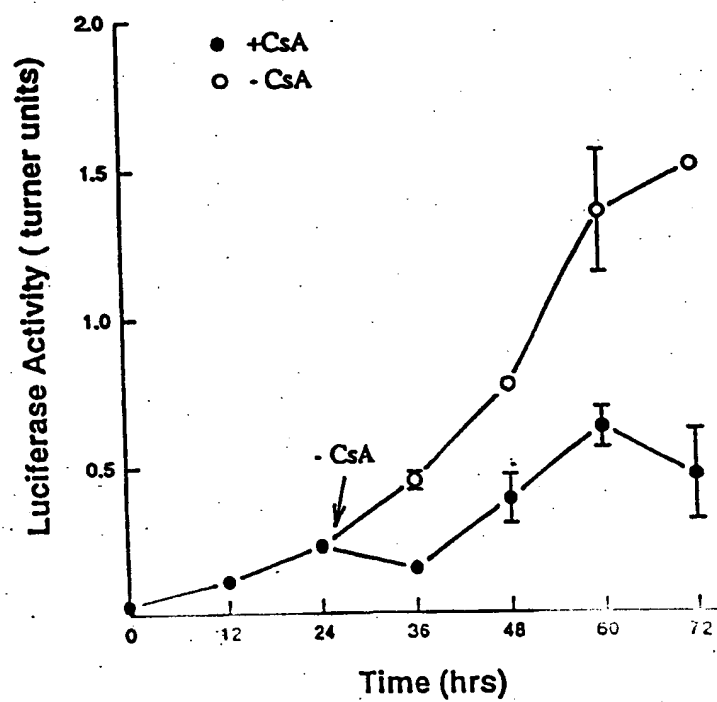


**FIGURE 15**

**FIGURE 16**



**FIGURE 17**

**FIGURE 18**

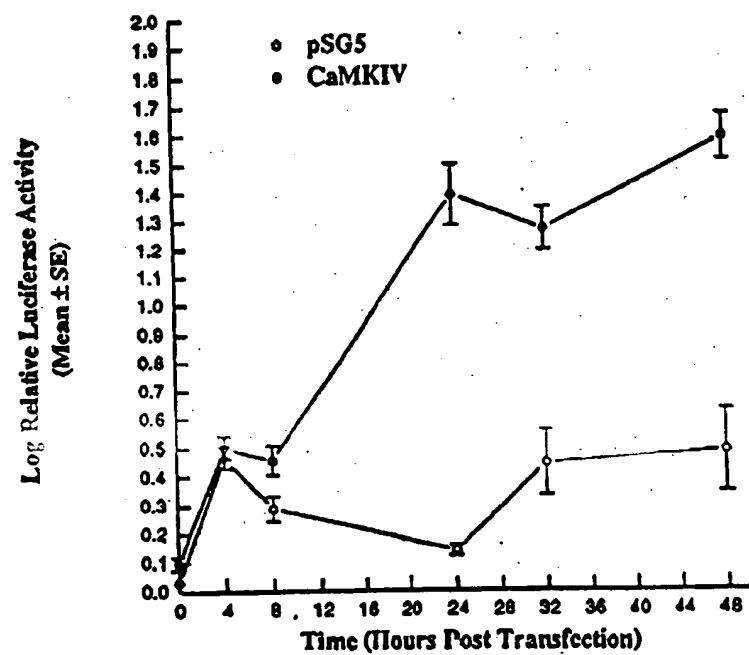
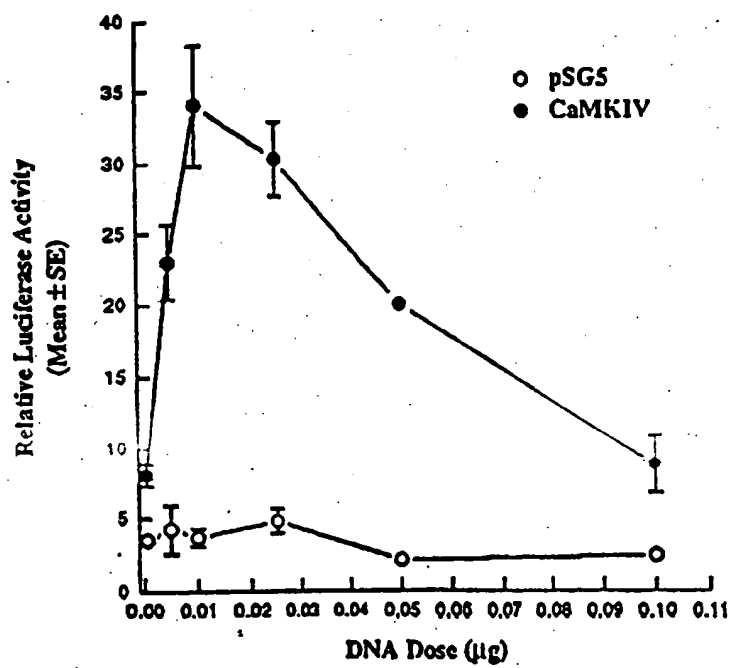
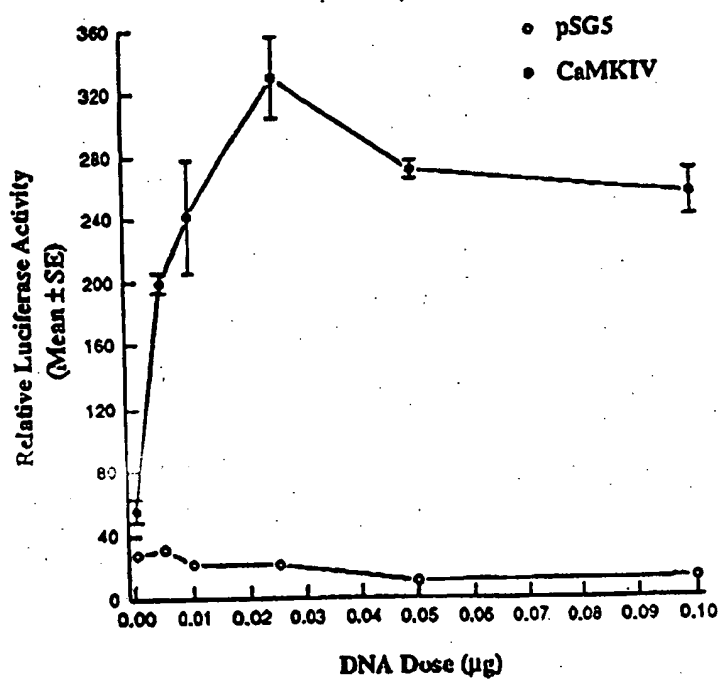
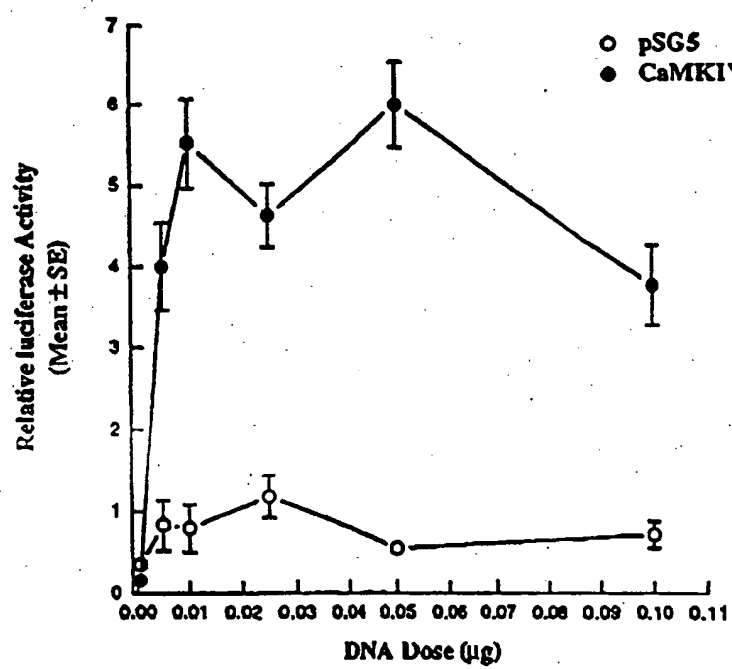
**FIGURE 19**

FIGURE 20



**FIGURE 21**

**FIGURE 22**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21988

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/00 A01K67/027 A61K48/00 A61K31/435 C07K14/58  
A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.        |
|------------|--|------------------------------|
| P, X       | MOLKENTIN, J.D. ET AL.: "A calcineurin-dependent transcriptional pathway for cardiac hypertrophy" CELL, vol. 93, no. 2, 17 April 1998, pages 215-228, XP002094705<br>NA US<br>see the whole document   | 1-6, 20, 37-39, 42-44, 47-54 |
| A          | HAVERICH, A. ET AL.: "Cyclosporin A and transplant coronary disease after heart transplantation: facts and fiction" TRANSPLANTATION PROCEEDINGS, vol. 26, 1994, pages 2713-2715, XP002094706<br>cited in the application<br>see the whole document | 2                            |

-/--



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

10 March 1999

Date of mailing of the international search report

23/03/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

Chambonnet, F

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21988

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| A          | <p>REID, C. J. &amp; YACOB, M. H.:<br/> "Determinants of left ventricular function<br/> one year after cardiac transplantation"<br/> BRITISH HEART JOURNAL,<br/> vol. 59, no. 4, April 1988, pages 397-402,<br/> XP002094707<br/> cited in the application<br/> see the whole document</p>          | 2                     |
| A          | <p>HARRIS, A. N. ET AL.: "A novel A/T-rich<br/> element mediates ANF gene expression<br/> during cardiac myocyte hypertrophy"<br/> JOURNAL OF MOLECULAR AND CELLULAR<br/> CARDIOLOGY,<br/> vol. 29, no. 2, February 1997, pages<br/> 515-525, XP002094708<br/> see page 523, paragraph DISCUS.</p>  | 1                     |
| A          | <p>HOEY, T. ET AL.: "Isolation of two new<br/> members of the NF-AT gene family and<br/> functional characterization of the NF-AT<br/> proteins"<br/> IMMUNITY,<br/> vol. 2, no. 5, May 1995, pages 461-472,<br/> XP002094709<br/> cited in the application<br/> see page 469, column 2</p>         | 1,3,4,<br>37,38       |
| A          | <p>KUDOH, S. ET AL.: "Angiotensin II<br/> stimulates c-jun NH2-terminal kinase in<br/> cultured cardiac myocytes of neonatal<br/> rats"<br/> CIRCULATION RESEARCH,<br/> vol. 80, no. 1, January 1997, pages<br/> 139-146, XP002094710<br/> cited in the application<br/> see the whole document</p> | 1                     |
| A          | <p>BOHELER, K.R. &amp; SCHWARTZ, K.: "Gene<br/> expression in cardiac hypertrophy"<br/> TRENDS IN CARDIOVASCULAR MEDICINE,<br/> vol. 2, no. 5, September 1992, pages<br/> 176-182, XP002096139<br/> see the whole document</p>  | 3,4                   |
| A          | <p>LEE, R.T. ET AL.: "Atrial natriuretic<br/> factor gene expression in ventricles of<br/> rats with spontaneous biventricular<br/> hypertrophy"<br/> JOURNAL OF CLINICAL INVESTIGATION,<br/> vol. 81, no. 2, February 1988, pages<br/> 431-434, XP002096140<br/> see the whole document</p>        | 37                    |



# INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 98/21988

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claims 1-21**  
**are directed to a method of treatment of the human/animal**  
**body, the search has been carried out and based on the alleged**  
**effects of the compound/composition.**
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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